Assessment of fluoride induced male reproductive disorders in adult Wistar rats and its amelioration by supplementation of vitamin C and E

Submission of 5000 Words synopsis

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Ph.D. registration No.: R-17RS18170107

Date: 14th August, 2018

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Synopsis (5000 words)

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Title of Thesis:	Assessment of fluoride induced male reproductive disorders in adult Wistar rats and its amelioration by supplementation of vitamin C and E
PhD registration Number with date:	R-17RS18170107dated 14th August, 2018
Enrolment Number with date:	PU/PHD/BIOS/2017/019 dated 20th September, 2017
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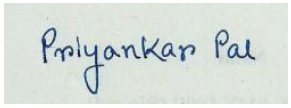

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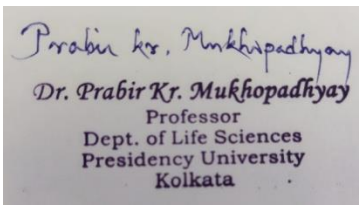

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Summary of Work

Title: Assessment of fluoride induced male reproductive disorders in adult Wistar rats and its amelioration by supplementation of vitamin C and E

1. Abstract

Fluoride is necessary for tooth and bone development, but excessive exposure causes system disorders along with reprotoxic consequences. The purpose of this study was to investigate the extent of fluoride toxicities in male reproductive system and its possible amelioration by vitamin C and E supplementation. To study the optimum dose of fluoride responsible for causing maximum reprotoxic effects, rats were divided into four groups. Control (group I) rats were received vehicle only and treated rats (group II, III, and IV) were administered sodium fluoride (NaF) orally at 10, 15, and 20 mg/kg/day doses, respectively, for 30 consecutive days. Histoarchitecture of testis and epididymis; epididymal sperm potential and spermatozoal DNA status; testicular oxidative status, activities of functional marker enzymes and DNA integrity were assessed. All these parameters were altered in a dose dependent manner. Based on these findings, it was determined that the minimal fluoride dosage responsible for maximum reprotoxic effects was 15 mg/kg/day and the further experiments were carried out with this dose. To study the protective role of vitamin C and E (VC&VE) against fluoride induced reproductive disorders, other sets of rats were procured and, in each time, they were divided into four groups. Group-I was the control; Group-II received NaF at 15 mg/kg/day dose; Group-III was provided with VC (200 mg/kg/day) and VE (400 mg/kg/day) plus NaF; Group-IV received only VC&VE for 30 consecutive days. Structure-function integrity of testis, epididymis and spermatozoa; oxidative status of testis and epididymis, testicular functional and steroidogenic status, apoptotic pathway of testis and epididymis, testicular inflammatory status along with spermatozoal apoptotic state were evaluated. All these parameters were altered due to fluoride treatment, while supplementation with VC&VE restored all the anomalies either to normal or near normal levels. The group IV rats did not exhibit any noticeable differences with the group I rats.

2. Introduction

Fluoride being an electronegative halogen element, derived from fluorine gas, never exists as a free state in nature. Being 13th most abundant element on the earth crust, fluoride prevails ubiquitously in the environment, combining with other elements, forming fluoride compounds, which are the constituents of minerals in rocks and soils[1]. Fluoride is also present in trace amount in mineralized tissues of the body like enamel, dentin and bone where it plays an indispensable role in enamel formation and bone mineralization[2]. Interestingly, inadequate intake of fluoride (less than 0.5 ppm) causes dental caries, lack of enamel formation and diminished bone mineralization, specifically among the children; whereas the excess consumption beyond the WHO permissible upper limit (1.5 ppm) leads to physiological complications, known as fluorosis[3]. Hence, unlike other elements fluoride exhibits self-contradictory nature and due to its harmful effects, it is also considered as a potential environmental toxicant. Although the beneficial impressions of fluoride are restricted to teeth and bone but the detrimental consequences are observed in teeth, bone as well as in soft tissues which are known as dental, skeletal and non-skeletal fluorosis respectively. The severity of fluorosis depends on the availability of fluoride in nature and degree of its exposure.

Both the natural reservoirs and anthropogenic activities are answerable for global fluoride contamination. Volcanic eruption, fluoride rich minerals and rocks, contaminated ground water and soil are natural sources of fluoride whereas dental products, fluoride containing pesticides, industrialization, refrigerants and fire extinguisher are the anthropogenic derivations[4]. The major source through which humans are exposed to fluoride is contaminated ground water where the level of fluoride is usually higher than that of surface water due to fluoride percolation from soil through leaching[5].

Globally, the countries which are prone to fluoride contamination include, China, Ethiopia, Tanzania, Mongolia, Sri Lanka, Kenya, Germany, Canada, Mexico, South Africa, Cameroon, Ghana, Malawi, India and East Punjab region of Pakistan. In India, 9 states show ground water fluoride level beyond 10 mg/L, which include Gujarat, Rajasthan, Delhi, Assam, Andhra Pradesh, Maharashtra, Madhya Pradesh, Karnataka and West Bengal (Dharmshaktu N 2013)[6]. As per the Public Health and Engineering (PHED) of West Bengal Government, about 225 villages from 42 blocks over 7 districts (Birbhum, Maldah, Bankura, Purulia, South 24 Parganas and North & South Dinajpur) are affected due to ground water contamination with fluoride[7].

Fluoride, after entering in the body undergoes metabolism through steps including absorption, distribution and renal excretion. About 40% of the ingested fluoride may be absorbed through the stomach in the form of hydrogen fluoride, where absorption is inversely related to the pH of stomach. Rest of the fluoride is then absorbed via upper small intestine in the form of fluoride ion. Although gastric absorption of fluoride in dependent on stomach pH, absorption through small intestine in pH independent and ionic fluoride enters through paracellular tight junctions present between the enterocytes. After absorption, fluoride is rapidly distributed throughout the body and plasma fluoride concentration starts to increase after 10 minutes of fluoride absorption. Peak plasma fluoride concentration reaches after 20-60 minutes of absorption whereas it takes 3-11 hours to reach the plasma fluoride in pre-digested level[8]. Most of the absorbed fluoride is taken up by the calcified tissues where fluoride reversibly bound and released back into plasma when plasma fluoride concentration falls. About 99% of retained fluoride found in the mineralized tissues specially in bone but dentin and enamel are also considered as fluoride reservoirs. Concentration of fluoride in bones is not uniform, as the cancellous bones exhibits higher fluoride concentration as compared to that of compact bones due to larger surface area. Dentin fluoride concentration is similar to that of bone while enamel fluoride concentration is lower. Deposition of fluoride in soft tissues depends on rate of blood flow to tissues. Renal excretion is responsible for fluoride removal from the body which depends on urinary pH[9].

Oxidative stress is considered as the underlying mechanism of fluoride toxicity and this has been experimentally established via/ through in-vitro as well as in-vivo studies involving several soft tissues of experimental animals. Fluoride increases the generation of superoxide anion which further augments the concentration of hydroxyl radical and hydrogen peroxide. Fluoride also enhances the generation of nitric oxide which reacts with the superoxide anions and forms peroxynitrite. These are recognized as potent reactive oxygen species (ROS) responsible for generation of oxidative stress. Fluoride also interferes the disulphide bond formation that leads to accumulation of misfolded proteins in the endoplasmic reticulum and causing endoplasmic reticulum stress (ER Stress). The activities of cellular antioxidant enzymes are interrupted by fluoride that further strengthen the damaging effects of ROS. Fluoride can also bind to the functional amino groups surrounding the active centres of enzymes involved in glycolysis and Krebs cycle and thereby inhibits cellular respiration[1]. Mitochondria is also a soft target of fluoride and the damaging consequences can be characterized by the loss of membrane integrity and inhibition of respiratory enzymes

activities[10]. Fluoride, by binding with DNA through covalent bond, affects DNA strand directly as well as indirectly through the generation of free radicals[11].

Fluoride can cross cellular membranes and penetrates to soft tissues and is responsible for causing disorders in different systems including hepatic, renal, digestive, respiratory, nervous, immune and also reproductive[12]. Available information clearly states on the fluoride mediated male reproductive anomalies following studies on animal model as well as on human populations[13]. As per the epidemiological report, Indian population, were suffering from long term skeletal fluorosis (ground water fluoride level shoots up to 14.5 mg/L) and were also facing from testosterone deficiency[14]. Male infertility among married peoples was also observed in high endemic areas of India having fluoride concentration 38.5 mg/L[15]. Apart from these, birth abnormalities and miscarriage were also reported in human populations of the fluoride contaminated areas[16]. Animal experimentations also divulged a number of disarrays in male reproductive system like impaired spermatogenesis, structure-function alterations of testis and tainted steroidogenesis[13]. Overproduction of ROS followed by augmented oxidative stress is the decisive reason of fluoride induced male reproductive disorders and spermatozoa are disposed to oxidative damages too much due to profusion of polyunsaturated fatty acids along with restricted antioxidant ability[17]. Earlier study also revealed that oxidative stress was a potential reason of male infertility and seminal ROS concentration was seen to be elevated in infertile men[18]. Surplus ROS concentration also causes several types of spermatozoal DNA damages like chromosome deletion, chromatin cross-linking as well as single and double stand DNA breakage. DNA damage and excess ROS are crucial mediators of apoptosis by persuading pro-apoptotic proteins like cytochrome C, caspase 9 and caspase 3[18]. Therefore, the intracellular events, like ROS generation, DNA damage and apoptosis are interlinked with one another. In this study, we have tried to find out the crosstalk within fluoride mediated oxidative stress, DNA damage and apoptosis of testis, epididymis and spermatozoa. Spermatogenesis, testicular steroidogenesis and maturation of semen are also under the regulation of few inflammatory mediators which include TNF-α and NF-κB[19], altered expression of which may lead to abnormal testicular and spermatozoal functions. We have also tried to find out testicular inflammatory responses after fluoride exposure.

There are two strategies for the protection of oxidative stress caused by environmental contaminants which include the reduction of exposure to pollutants and increase of antioxidant defence[20]. Likewise, toxicity studies are intended to find out the management approaches

against the risk of environmental pollutants. Researchers have used several natural products having antioxidant properties against oxidative damages which are curcumin, leutin, beta carotene, rutin, quercetin, naringenin, resveratrol, ascorbic acid, α-tocopherol etc[21]. Ascorbic acid or vitamin C (VC) is a potent water-soluble ROS scavenger which exerts its effects in the aqueous environment of cell membranes while α-tocopherol or vitamin E (VE) being a hydrophobic peroxyl radical scavenger acts as chain breaking antioxidant. Recent research further states that beyond antioxidant property, VC also acts as cofactor of enzyme which promotes DNA demethylation and directly influence genomic stability[22]. VE controls the activities of enzymes (regulates energy, lipid, and glucose metabolism) involved in the signal transduction and transcription by direct and indirect mechanisms and thus alters gene expression[23]. Interaction between VC and VE has led to idea about recycling of the VE where antioxidant capability of oxidized VE is restored by VC[24]. Therefore, it may be assumed that combined application of these vitamins could be more effective in toxicity management compared to the individual applications. Literature also states that co-administration of vitamin C and E exhibits more protective role against oxidative stress, hematological and histopathological alterations caused by copper toxicity in broiler chickens than individual application of such vitamins[25]. Another recent report reveals that the protective ability of co-administration of Vitamin C and E against cadmium induced liver and kidney damages, oxidative stress and apoptosis where the chosen supplementation showed cadmium chelation and anti-apoptotic activities[26]. Our lab has also reported that combined application of VC and VE has defensive efficacy against arsenic mediated hematological and hepatic anomalies[27]. Still there is dearth of information regarding the role of these vitamins against fluoride mediated testicular, epididymal and spermatozoal anomalies at the genomic and proteomic levels.

On the basis of these background information, I have tried to find out the extent of protective ability of VC and VE against fluoride mediated testicular, epididymal and spermatozoal abnormalities. Apart from structural, functional and oxidative damages, I have also focused on apoptosis as well as testicular inflammatory responses.

3. Aim and objectives

Aim: Whether vitamin C and E can alleviate the detrimental effects of fluoride in male reproductive system.

Objectives:

1. Selection of optimum dose of fluoride responsible for causing reproductive disorders by dose dependent studies.
2. Assessment of fluoride induced testicular toxicity and its amelioration by dietary supplements.
3. To study the extent of damages in epididymal segments and subsequent protection.
4. Assessment of signaling pathway/s involved in the mitigation of fluorotoxicity in reproductive organs by the supplement.

4. Materials and methods

4.1 Selection of animals and treatment

Objective 1: Thirty-two adult male Wistar rats weighing 120±10 gm were selected for experiments and were maintained under standard laboratory condition (14 hr light: 10 hr dark, 25±2 ˚C temperature) having free access to feed (Table 1) and water. The given standard diet was composed of 71% carbohydrate, 18% protein, 7% fat, 4% salt mixture and vitamins. After 10 days of quarantine period, animals were equally divided into four groups. Group I was control (vehicle fed), Group II, III and IV were treated with aqueous solution of sodium fluoride (NaF) by gavaging at dose of 10, 15 and 20 mg/kg/day respectively. Treatment schedule was continued for 30 consecutive days.

Objective 2, 3 and 4: After selection of the optimum dose of NaF further experimental design was conducted to study the protective ability of chosen supplement. For fulfilment of these objectives, thirty-two adult male Wistar rats weighing 120±10 gm were selected. Laboratory condition and diet were similar to that of objective 1. After 10 days of quarantine period animals were equally divided into four groups. Group I was control (vehicle fed), Group II was treated with NaF at a dose of 15 mg/kg/day. Group III was supplied with VC (200 mg/kg/day) and VE (400 mg/kg/day) along with NaF of aforementioned dose. Group IV was provided with only VC&VE at same dose like Group III. Treatment schedule was continued for 30 consecutive days.

Same animal models were repeated to achieve the target results. The said animal model and the follow up experiments were conducted as per the ethical guidelines suggested by the Institutional Animal Ethical Committee (Sanction No. PU/IAEC/PM/20).

Table 1: Composition of normal diet per 100 gm

Normal diet			
Components	Composition	gm %	Energy
Proteins	Whole wheat flour	3.62	4.178 kcal/g
	Roasted Bengal gram flour	4.38	
	Casein	10	
	Total	18	
Carbohydrates		71	
Lipids		7	

Minerals and vitamins		4	

4.2 Euthanasia and collection of tissues and/or samples

After 30 days of treatment period, animals from each group (for fulfilment of all the objectives) were euthanized following overnight fast. Immediately, blood was collected for serum isolation from which hormonal assay was performed. Organs like testes, epididymis, prostate and seminal vesicle were dissected, cleaned properly and weighed. Testes and epididymis (caput and cauda regions) from either side were isolated for histology and scanning electron microscopy (SEM) whereas such organs from other side were stored in –80 °C refrigerator for remaining studies. Spermatozoa were isolated immediately from both caput and cauda regions of dissected epididymis for different studies.

4.3 Body weight and gravimetric index

Initial and final body weight as well as gross organ weight of rats from all groups were noted. Relative organ weight (gravimetric index) was calculated from gross organ weight and body weight.

4.4 Histology of testes and epididymis

For histological studies, testes and epididymis from the rats of all groups were fixed in Bouin's solution followed by embedding in paraffin wax (56–58 °C). Five micrometer (μ) thick sections were cut by well precision microtome (Optex brand, Pink Pearl Corporation, Osaka, Japan) and stained with haematoxylin-eosin (H/E). Photomicrographs of stained slides were taken at 100X and 400X magnification of microscope (Zeiss, Thornwood, NY, USA).

4.5 Histomorphometry analysis from testes

For histomorphometric analysis, seminiferous tubular profile (average of minimum 15 observations from each rat of every group) were microscopically (Zeiss, Thornwood, NY, USA) captured and measured by using Progress Capture Pro 2.5 software (GENOTYPIC Optical Systems GmBH, Jena, Germany). Seminiferous tubule diameter, seminiferous epithelial height and luminal diameter were measured. Leydig cell nuclear area was also assessed.

4.6 Scanning electron microscopy of testes and epididymis

Scanning electron microscopy (SEM) was performed from both testes and epididymis. The tissues were fixed in 2.5% glutaraldehyde, sliced and dehydrated following the protocol of Romero et al[28]. Then samples were coated with 5 mm of gold and images were captured by using scanning electron microscope (Zeiss EVO-MA 10).

4.7 Assessment of spermatozoal potential and evaluation of morphology

Spermatozoal features were assessed after isolation from epididymis[29] (cauda for objective 1; both caput and cauda for rest objectives). Epididymis was placed petri dish containing 1X PBS at 37 ^{o}C, then incised properly so that spermatozoa were released in the medium[30]. This sperm suspension was used for evaluation of different parameters.

In Neubauer haemocytometer chamber 10 µL sperm suspension was charged and counting was performed under 400X magnification of microscope (Zeiss, Thornwood, NY, USA)[31]. This procedure was repeated three times from each rat of all groups which was considered as a single data. Thus other 7 average values were resulted from rest 7 rats of each group

Same sperm suspension was used for sperm motility assay and 10 µL of sample was dispended on grease free slide and mounted with clean coverslip. Then counting was performed[31] under 400X magnification of microscope (Zeiss, Thornwood, NY, USA). As per different movement pattern, sperm motility can be categorized as progressive motile spermatozoa (PMS), nonprogressive motile spermatozoa (NPMS) and non-motile spermatozoa (NMS). After addition of PMS and NPMS we got the total number of motile spermatozoa and the data were expressed in percentage. This procedure was repeated three times from each rat of all groups which was considered as a single data. Thus other 7 average values were resulted from rest 7 rats of each group.

Spermatozoal viability was evaluated with the help of eosin-nigrosin staining[31]. Within eppendrof 40 µL spermatozoal suspension was taken and then 40 µL each of eosin Y and nigrosin were added separately. After proper mixing, the smear was drawn from this mixture on clean slide and photographs were taken from air-dried slides under 400X magnification of microscope (Zeiss, Thornwood, NY, USA). Spermatozoa having unstained heads were considered as viable, while pink stained heads were considered as nonviable and numbers of both viable as well as nonviable spermatozoa were expressed in percentage. From each rat 3 slides were prepared and the respective counts were done and

their average was treated as a single data. Thus other 7 average values were resulted from rest 7 rats of each group.

Evaluation of spermatozoal morphology and sperm abnormalities were also evaluated from the slides stained with eosin-nigrosin and photographs were taken under 400X magnification of microscope (Zeiss, Thornwood, NY, USA). From each rat 3 slides were prepared and the respective counts were done and their average was treated as a single data. Thus other 7 average values were resulted from rest 7 rats of each group.

Assessment of spermatozoal plasma membrane functional integrity was performed by hypo-osmotic swelling (HOS) test[32]. In eppendrof 100 µL sperm suspension was taken in which 1 mL hypo-osmotic solution (prepared by addition of 7.35 g sodium citrate and 13.51 g fructose in 1 L distilled water) was added. Then the mixture was incubated at 37 ºC for 1 hour. After incubation the mixture was spread in slide for the observation of coiled tail at 400X magnification of microscope (Zeiss, Thornwood, NY, USA). Spermatozoa with coiled tail were considered as HOS+ve and uncoiled tail were considered as HOS-ve. Numbers of both types were expressed in percentage. This procedure was repeated three times from each rat of all groups which was considered as a single data. Thus other 7 average values were resulted from rest 7 rats of each group

4.8 Scanning electron microscopy of spermatozoa

Spermatozoal morphology was evaluated with the help of scanning electron microscopy[33]. Sperm suspension was fixed within 2.5% glutaraldehyde for an hour followed by smeared on clean cover slip. The dried smear of spermatozoa was then coated with gold and observed under scanning electron microscope (Zeiss EVO-MA 10).

4.9 Assessment of oxidative stress from testis and epididymis

Testicular and epididymal oxidative stress was measured by evaluating enzymatic and non-enzymatic parameters through biochemical analysis using spectrophotometer. Lipid peroxidation was assessed by measuring fluorescent red adduct formed by reaction between thiobarbituric acid (TBA) with the malondialdehyde (MDA), which is the end product following oxidation of omega-3 and omega-6 fatty acids[34]. Protein carbonyl (PC) content was measured from tissue homogenate which is formed after protein oxidation following a reaction with the 2,4-dinitrophenylhydrazine (DNPH)[35]. Reduced glutathione (GSH)

content from tissue homogenate was measured by the reaction catalyzed by reducing 5,5-dithiobis-2-nitrobenzoate to 2-nitro-5-thiobenzoate[36]. Activity of superoxide dismutase (SOD) was measured following autooxidation of haematoxylin as per the method of Martin et al[37]. Catalase activity was assessed following catalytic breakdown of H_2O_2 by this enzyme[38]. Glutathione S transferase (GST) activity was measured based on its catalyzing effects on conjugation reaction with the glutathione following the protocol of Boyland et al[39]. Glutathione peroxidase (GPx) activity was measures based on the method of Flohe and Gunzler[40]. Each assay was repeated three times from each rat of all groups.

4.10 Activities of testicular functional marker enzymes

The activities of testicular acid phosphatase (ACP)[41] and alkaline phosphatase (ALP)[42] were measured spectrophotometrically following standard protocol. Testicular lactate dehydrogenase (LDH) activity was measured following the protocol of kit purchased from span diagnostics (Mumbai, India). Each assay was repeated three times from each rat of all groups.

4.11 Assessment of testicular steroidogenic activities and measurement of serum gonadotropins

Testicular steroidogenic activity was evaluated from testicular tissue homogenate by assessing the levels of two key regulatory marker enzymes *i. e.* Δ^5,3β-hydroxysteroid dehydrogenase (3β-HSD)[43] and Δ^5,17β-hydroxysteroid dehydrogenase (17β-HSD)[44].

Levels of serum follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were measured following kit protocol obtained from Elabscience, Houston, Texas. Each assay was repeated three times from each rat of all groups.

4.12 DNA fragmentation assay from testis and epididymis

Genomic DNA from testis and epididymis was extracted by the phenol/chloroform-/isoamyl alcohol following standard protocol[45,46]. Purity of DNA was determined by assessing the ratio of the absorbance at 260/280 nm. For each sample 3μg of DNA were loaded with DNA marker in 0.8% agarose gel. Then samples were run at 5V for the 5 minutes followed by increasing to the 100V and documented in gel documentation system (Bio-Rad).

4.13 Single cell gel electrophoresis from testis

The tissues were minced and suspended (2 mg/mL) in the cold Hank's balanced salt solution (HBSS). The suspension was then allowed for 15 min to settle down. Cell suspension (5–10 lL) was then mixed with 75 lL 1% low melting point agarose and embedded in microscope slide coated with thin 1% normal melting point agarose. DNA was allowed to unwind in alkaline condition. Electrophoresis was performed at 280 mA and 24 V for 20 min under alkaline condition (300 mM NaOH and 1 mM EDTA, pH > 13). Damaged DNA or broken DNA fragments undergoing electrophoresis migrate away from the nucleus and migration is inversely proportional to the size of the fragments. The slides were then neutralised with neutralising buffer followed by stained with fluorescent dye ethidium bromide (2 lg/mL). DNA 'comet' was visualised by fluorescence microscope. The DNA damage was evaluated by the length of the comet's tail by Image J[47].

4.14 Spermatozoal ROS generation by 2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA)

Levels of intracellular H_2O_2 in spermatozoa isolated from caput and cauda epididymis were measured by using fluorescent probe, DCFH-DA. Spermatozoal number was adjusted to one million per mL and DCFH-DA was dissolved in DMSO at 5mM concentration. Samples were stored in dark condition for 30 minutes and analyzed in flow cytometer (BD FACS Verse).

4.15 Estimation of spermatozoal maturity and DNA integrity

Maturity of spermatozoa was evaluated by assessment of histone and protamine contents in sperm nuclei with the help of aniline blue (AB) staining[48]. Immature spermatozoa having histone rich nuclei reacts positively with aniline blue and head of the spermatozoa turned blue (AB+ve) while mature spermatozoa with protamine rich nuclei are non-reactive to aniline blue and remain unstained (AB-ve). The number of AB+ve and AB-ve was counted under microscope (Zeiss, Thornwood, NY, USA) and data was presented in percentage.

Integrity of spermatozoal DNA was assessed by staining of the air-dried smears of spermatozoa with acridine orange (AO) dye[49]. Spermatozoa with double stranded native DNA fluoresced green while those of denatured DNA fluoresced yellow to red for meta-chromatic shifting. Percentage of the native intact DNA and denatured DNA were calculated under fluorescent microscope (AXIO Imager M1 fluorescence microscope, Carl Zeiss, Germany).

4.16 Estimation of spermatozoal apoptosis

During normal situation, phosphatidyl serine, one important plasma membrane phospholipid located at inner leaflet of plasma membrane. Apoptosis of epididymal spermatozoa caused translocation of phosphatidylserine across plasma membrane which was evaluated by flow cytometry (BD FACSVerse)[50,51]. FITC Annexin V/Dead Cell Apoptosis kit obtained from Invitrogen was used to detect spermatozoal apoptosis.

4.17 RNA extraction and synthesis of cDNA

RNA was extracted from the testicular and epididymal (cauda) tissues by Trizol followed by mixed mixing with phenol and chloroform at 13000×g. RNA pellet was then washed with cold ethanol, air dried and dissolved in 1X TAE buffer. Quality of extracted RNA was measured by spectrophotometer (NanoDrop ND-2000, USA). Pure RNA sample with A260/A280 ratio of 1.8:2 was utilized for synthesis of cDNA which was performed by using 1 µg of RNA following kit protocol (Biobharti Pvt. Ltd., India). Synthesized cDNA was then stored in −80 °C refrigerator for subsequent analysis.

4.18 Real-time PCR using SYBER green

Quantitative real-time PCR was performed by using Bio-Rad CFX96 (C1000) Touch Real-time PCR (Bio-Rad, USA) following the protocol of Mahadevi S[52]. Quantitative analysis was performed by determining the increased fluorescence light associated with SYBR Green bonding. For fulfilment of the assay 1.5 µl of cDNA, 2 µl of primer (1 µl of each forward and reverse), 5 µl of SYBR Green Master Mix and 1.5 µl of deionized water were mixed. Primer design of Bax, Bcl-2, Bad, Bak, TNF-α and β-actin were done by using Primer3 tool (Table 2). The amplification and thermal cycle of the selected genes were as follows: initial denaturation for 5 seconds at 95 °C followed by 40 cycles for 30 seconds at 61 °C and final extension at 75 °C for 20 seconds. Relative expressions of mRNA were calculated by using ΔΔCt method. Relative fold increase was calculated by the following equation: $RFI = 2^{-\Delta\Delta CT}$.

Table 2: Primer sequences used for real-time PCR

Primer Sequence (5'-3')				
Target Gene	Forward	Reverse	Size (bp)	Product size
Bax	AACATGGAGCTGCAGAGGAT	CCAGTTGAAGTTGCCGTCTG	22	105 bp

BCl-2	GTGGACAACATCGTCCTGTG	GAGCAGCGTCTTCAGAGACA	20	156 bp
Bad	TTAGCCCTTTTCGAGGACGC	TACGTCAGCTCTTTCCCTTG	21	363 bp
Bak	TTCTCGGGCTAAAAGGCGAC	CTTGTCACCTGCCTGACTGCT	21	174 bp
TNF-α	ATCGGTCCCAACAAGGAGGA	CCGCTTGGTGGTTTGCTACG	20	139 bp
β-actin	CGCGAGTACAACCTTCTTGC	CGTCATCCATGGCGAACTGG	20	70 bp

PCR- polymerase chain reaction

4.19 Immunoblotting of selected apoptotic marker proteins from testis and epididymis

Immune blots from testicular and epididymal (cauda) samples were prepared following the protocol of Biswas P, 2019[53]. Blots were incubated overnight with respected antibodies (Bax, Bcl2, Nrf2, cleaved caspase 3, cleaved caspase 9, NF-κβ, and TNF-α) at 4 °C. Rabbit anti-β-actin antibody was used as a loading control. After washing, blots were incubated with secondary antibody (conjugated with alkaline phosphatase) for 2 hours at 25 °C. Immune-reactive proteins were detected by staining the membrane with NBT-BCIP substrate solution. Band intensity was measured by ImageJ software (NIH, USA; Version 1.52a). Relative concentration of each protein was calculated by considering the value of control as one[54].

4.20 Estimation of fluoride content in testis

Testicular fluoride content was estimated by digesting the tissues through alkali fusion process. Fluoride concentration was measured electrochemically by ion-selective electrode (ISE) meter (Thermo Scientific Orion Star A214, USA) combined with the fluoride ion-selective electrode (Orion ISE model no. 9609BNWP)[55].

4.21 Statistical analysis

The data were presented as mean ± standard error of the mean (SEM) and one -way ANOVA followed by the Tukey's HSD post hoc analysis was done to check statistical difference between the parameters of studied groups. $p<0.05$ was considered as the lowest significant. Statistical analysis was performed using statistical program packages SPSS version 20.0 (SPSS Inc., Chicago, USA).

4.22 Heat map analysis

Heat map analysis was done by the help of TBtools software where the respective mean values of data were taken.

5. Results

5.1 Objective 1

For fulfilment of the first objective, different parameters of testis, epididymis and spermatozoa were studied, such as histology of testis and epididymis (caput and cauda), levels of enzymatic and non-enzymatic oxidative stress markers from testis, activities of testicular functional marker enzymes, potential of spermatozoa isolated from cauda and DNA damage of testis and spermatozoa. The results are presented here.

5.1.1 Effect of NaF on body weight and gravimetric index of reproductive organs

Treatment with NaF caused significant decrease ($p<0.01$) of body weight which was observed in group III and IV. NaF also caused reduction of gravimetric index but most were insignificant (Table 3). Significant reduction of testicular gravimetric index was observed in group III and IV ($p< 0.01$ in both cases). No marked reduction in gravimetric index was observed in case of epididymis, prostate and seminal vesicle.

Table 3: Effect of graded doses of NaF on body weight and gravimetric index of primary as well as accessory reproductive organs of adult rats

Animals	Difference in body weight (final- initial) %	Gravimetric index (g %)			
		Testis	Epididymis	Prostate	Seminal vesicle
Group I	7.848±3.003	0.801±0.015	0.233±0.015	0.047±0.003	0.35±0.047
Group II	5.426±4.301	0.705±0.017	0.23±0.008	0.042±0.003	0.33±0.020
Group III	-7.140±2.236^{a**b*}	0.643±0.027^{a**}	0.225±0.012	0.039±0.005	0.29±0.017
Group IV	-7.728±4.154^{a**b*}	0.632±0.047^{a**}	0.228±0.014	0.036±0.001	0.28±0.017

Values are presented as mean±SEM, n = 8 and comparison is made by *Tukey's test*.
a = group I vs group II, III & IV; b = group II vs group III & IV. *p<0.05, **p<0.01.

5.1.2 Changes of testicular histology after application of NaF

Structural anomalies of testis were noted along increasing dose of NaF (Figure 1). Testicular histology from control rats was characterized by compact orientation of seminiferous tubules within testicular parenchyma, presence of successive developmental stages of spermatogonial cells within seminiferous tubule and intact Leydig cells between seminiferous tubules. Though no noticeable alterations were noted in group II rats but marked degenerative changes were observed in group III and IV, characterized by disorganization of seminiferous tubule, absence of successive developmental pattern of spermatogonial cells and disintegrated Leydig cells. Damages were severe in group IV.

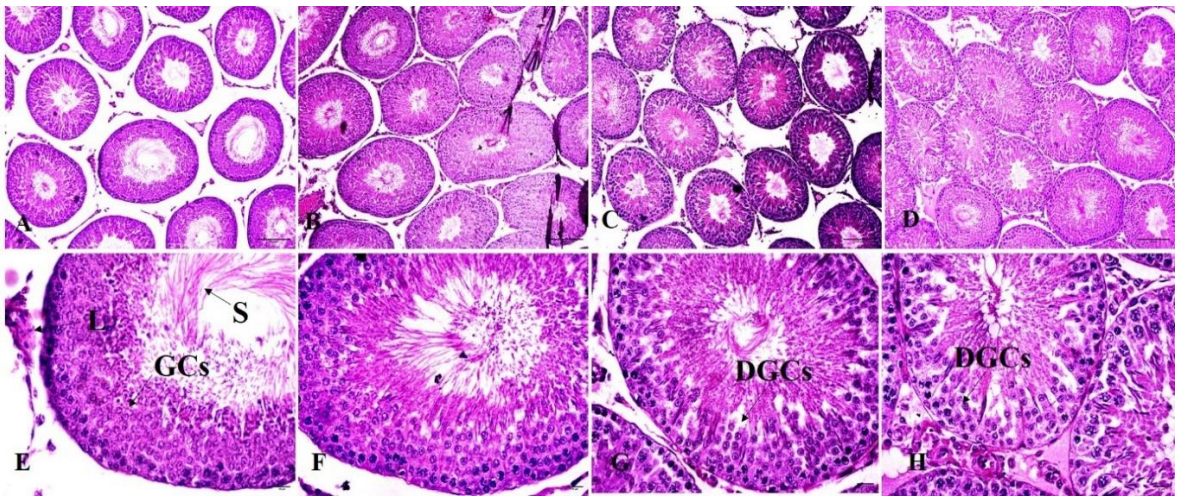


Figure 1: Representative images of testicular histoarchitecture evaluated by H/E staining. A & E are representative tissue sections at 100X and 400X magnification of group I (control) rats; B & F are tissue sections at 100X and 400X magnification of group II (NaF 10mg/Kg) rats; C & G are tissue sections at 100X and 400X magnification of group III (NaF 15mg/Kg) rats; D & H are photomicrographic representation of group IV (NaF 20mg/Kg) rats at 100X and 400X magnification. Germ cells (GCs), Leydig cells (L), spermatozoa (S), distorted germ cells (DGCs).

5.1.3 Histological alterations of epididymis

Altered histology was also observed in epididymis in a dose dependent manner, more particularly the adverse effects were noted in case of cauda epididymis compared to caput.

Histological observations of initial segment of epididymis (caput) from group I were defined as dense layer of stereocilia on pseudostratified columnar epithelial layer and presence of masses of spermatozoa within the lumen. No noticeable alterations were observed in group II

and III rats whereas degenerative changes were noted in group IV characterized by thinning of pseudostratified epithelium, partially bald appearance of stereocilia and decreased sperm concentration in lumen (Figure 2).

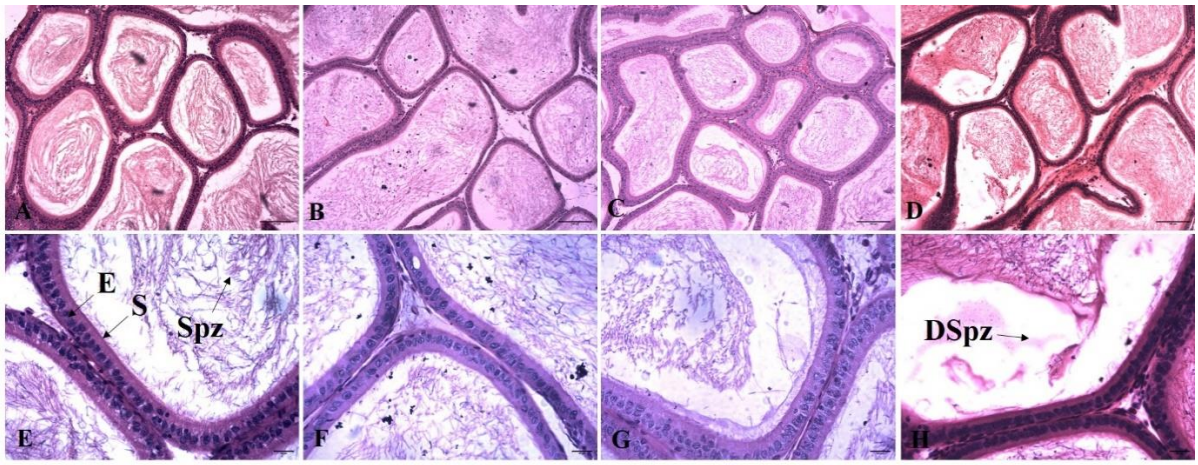


Figure 2: Representative images of epididymal (caput) histoarchitecture evaluated by H/E staining. A & E are representative tissue sections at 100X and 400X magnification of group I (control) rats; B & F are tissue sections at 100X and 400X magnification of group II (NaF 10mg/Kg) rats; C & G are tissue sections at 100X and 400X magnification of group III (NaF 15mg/Kg) rats; D & H are photomicrographic representation of group IV (NaF 20mg/Kg) rats at 100X and 400X magnification. Epithelial lining (E), stereocilia (S), spermatozoa (Spz), decreased spermatozoa (DSpz).

Histological features of last region (cauda) of epididymis from control rats were characterized by presence of lobules lined with pseudostratified epithelium and presence of stereocilia in luminal surface of epithelium. Luminal space is also filled with abundance of spermatozoa. Fluoride toxicity caused thinning of epithelium lining, partial absence of stereocilia and decreased luminal sperm concentration. All such damaging changes were observed in dose dependent manner, specifically in group III and IV (Figure 3).

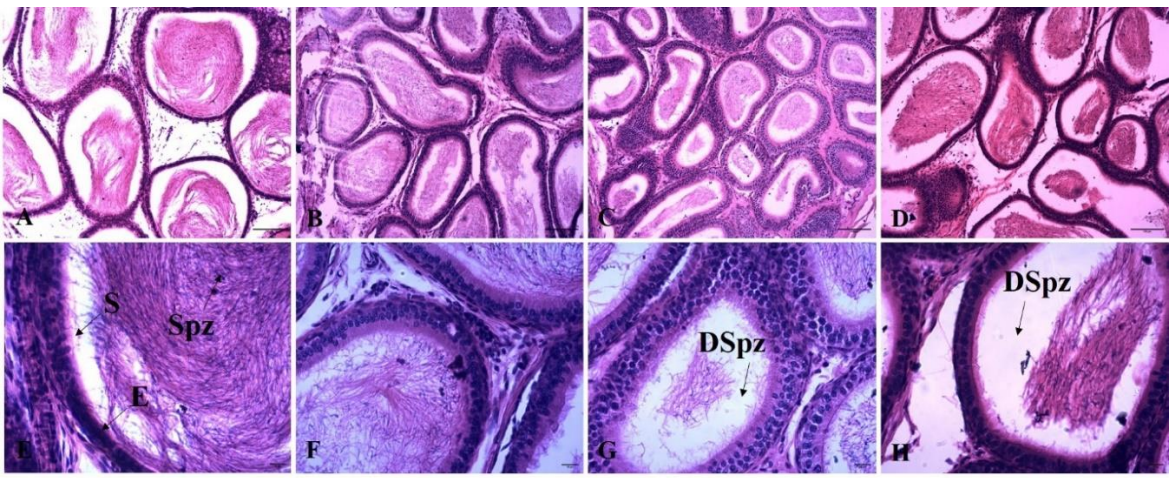


Figure 3: Representative images of epididymal (cauda) histoarchitecture evaluated by H/E staining. A & E are representative tissue sections at 100X and 400X magnification of group I (control) rats; B & F are tissue sections at 100X and 400X magnification of group II (NaF 10mg/Kg) rats; C & G are tissue sections at 100X and 400X magnification of group III (NaF 15mg/Kg) rats; D & H are photomicrographic representation of group IV (NaF 20mg/Kg) rats at 100X and 400X magnification. Epithelial lining (E), stereocilia (S), spermatozoa (Spz), decreased spermatozoa (DSpz).

5.1.4 Effect of NaF on spermatozoal potential

Marked reduction of epididymal (cauda) sperm potential was observed in a dose dependent way after application of graded doses of NaF (Table 4). Spermatozoal number was decreased significantly ($p< 0.001$ in all cases) in all treated groups having more pronounced effects in group IV compared to group II and III ($p<0.001$). Spermatozoal motility decreased significantly in a dose dependent way ($p< 0.001$ within group I, II, III, and IV). Percentage of viable spermatozoa decreased remarkably in group III and IV ($p< 0.001$) having marked differences among treated groups ($p< 0.001$). Integrity of spermatozoal plasma membrane was reduced in dose dependent manner ($p< 0.001$ within group I, II, III, and IV).

Table 4: Effect of graded doses of NaF administration on spermatozoal status of adult rats

Animals	Sperm parameters			
	Count (x 10^6)	Motility (%)	Viability (%)	HOS +ve (%)
Group I	109.93±5.835	88.91±1.937	79.10±1.608	79.12±1.787
Group II	81.93±3.976^{a***}	73.29±2.795^{a***}	74.05±1.419	65.87±1.444^{a***}
Group III	80.71±1.430^{a***}	48.25±2.415^{a***b***}	59.85±1.837^{a***b***}	52.87±1.381^{a***b***}
Group IV	50.73±1.229^{a***b***c***}	37.08±1.395^{a***b***c***}	35.40±1.790^{a***b***c***}	37.37±1.700^{a***b***c***}

Values are presented as mean±SEM, n = 8 and comparison is made by *Tukey's test*.
a = control (group I) vs treated groups (II, III & IV); b = group II vs group III & IV; c = group III vs group IV. ***p<0.001. HOS- Hypoosmotic swelling.

5.1.5 Effect of NaF on testicular oxidative stress

Expressions of testicular enzymatic and non-enzymatic oxidative stress markers were altered due to NaF exposure (Table 5 & Figure 4). Marked reduction of SOD activity was noted in all treated groups ($p<0.001$). Catalase activity decreased meaningfully in all treated groups ($p<$

.01 in group II; p< .001 in group III and IV). Declined GST activity was seen in all treated groups (p< .001) with maximum reduction in group IV. Marked reduction in GPx activity was observed in all treated groups (p< 0.001). MDA level enhanced significantly in group III (p< .01) and IV (p< .001). Enhanced PC content was noted in all treated groups (p< 0.001). Level of GSH was also seen to decrease significantly in all treated groups (p< 0.001) with respect to control.

Table 5: Alterations of testicular oxidative stress parameters by graded application NaF in adult rats

Animals	Oxidative stress parameters						
	Enzymatic				Non enzymatic		
	SOD (unit/mg tissue)	**Catalase (µM H_2O_2 consumed/mg tissue/min)**	**GST (µM NADPH consumed/min/mg protein)**	**GPx (nM NADPH oxidized/mg tissue/min)**	**MDA (nM/mg tissue)**	**PC (µM/mg protein)**	**GSH (µM/mg tissue)**
Group I	15.72±1.753	29.59± 2.599	718.82± 13.601	0.174± 0.005	8.81± 1.649	24.90± 0.864	606.74±11.346
Group II	6.15± 1.352^{a***}	22.14± 1.423^{a**}	425.33± 78.903^{a***}	0.052± 0.011^{a***}	15.72± 1.340	49.31± 4.431^{a***}	524.64±6.451 ^{a***}
Group III	4.08± 1.162^{a***}	14.64± 1.280^{a***b**}	389.00± 84.546^{a***}	0.043± 0.006^{a***}	21.65± 2.103^{a**}	60.85± 4.714^{a***}	402.82±8.542 ^{a***b***}
Group IV	2.02± 0.323^{a***}	10.21± 0.947^{a***b***}	140.39± 2038^{a***b***c**}	0.038± 0.010^{a***}	32.30± 3.612^{a***b***c**}	67.18± 5.604^{a***}	374.19±6.190 ^{a***b***}

Values are presented as mean ± SEM, n = 8 and comparison is made by *Tukey's test*. SOD-Superoxide dismutase; GST-Glutathione S-transferase; GPx-Glutathione peroxides; MDA-Malondialdehyde; and PC-Protein carbonyl; GSH- Reduced glutathione. a = control (group I) vs treated groups (II, III & IV); b = group II vs group III & IV; c = group III vs group IV. **p <0.01; ***p <0.001.

5.1.6 Effect on testicular functions by application of NaF

Activities of testicular functional marker enzymes were declined due to exposure to NaF (Table 6 & Figure 4). Notable decrease of ACP activity was seen in group III (p< .05) and group IV (p< .01). ALP activity was also diminished significantly in group III and group IV (p< .05). Level of LDH activity was declined meaningfully in all treated groups (p< .05 in group II and p< .001 in group III and IV) having variation between groups (p< .001 with group II and both group III and IV).

Table 6: Alterations of testicular functional parameters with graded doses of NaF in adult rats

Animals	Functional parameters of testis		
	ACP (µg of PNP liberated/ mg of tissue/ hr)	ALP (µg of PNP liberated/ mg of tissue/ hr)	LDH (unit/mg protein)
Group I	4.76±0.211	4.48±0.141	2.21±0.098
Group II	4.35±0.099	4.2857± 0.224	1.86±0.060^{a*}
Group III	3.71±0.344^{a*}	3.23±0.413^{a*}	1.01±0.047^{a***b***}
Group IV	3.42±0.217^{a**b*}	3.59±0.253^{a*}	0.87± 0.077^{a***b***}

Values are presented as mean ± SEM, n=8 and comparison is made by *Tukey's test*. ACP-Acid phosphatase; ALP-Alkaline phosphatase; LDH-Lactate dehydrogenase. a = control (group I) vs treated groups (II, III & IV); b = group II vs group III & IV. * p <0.05, ** p <0.01, *** p <0.001.

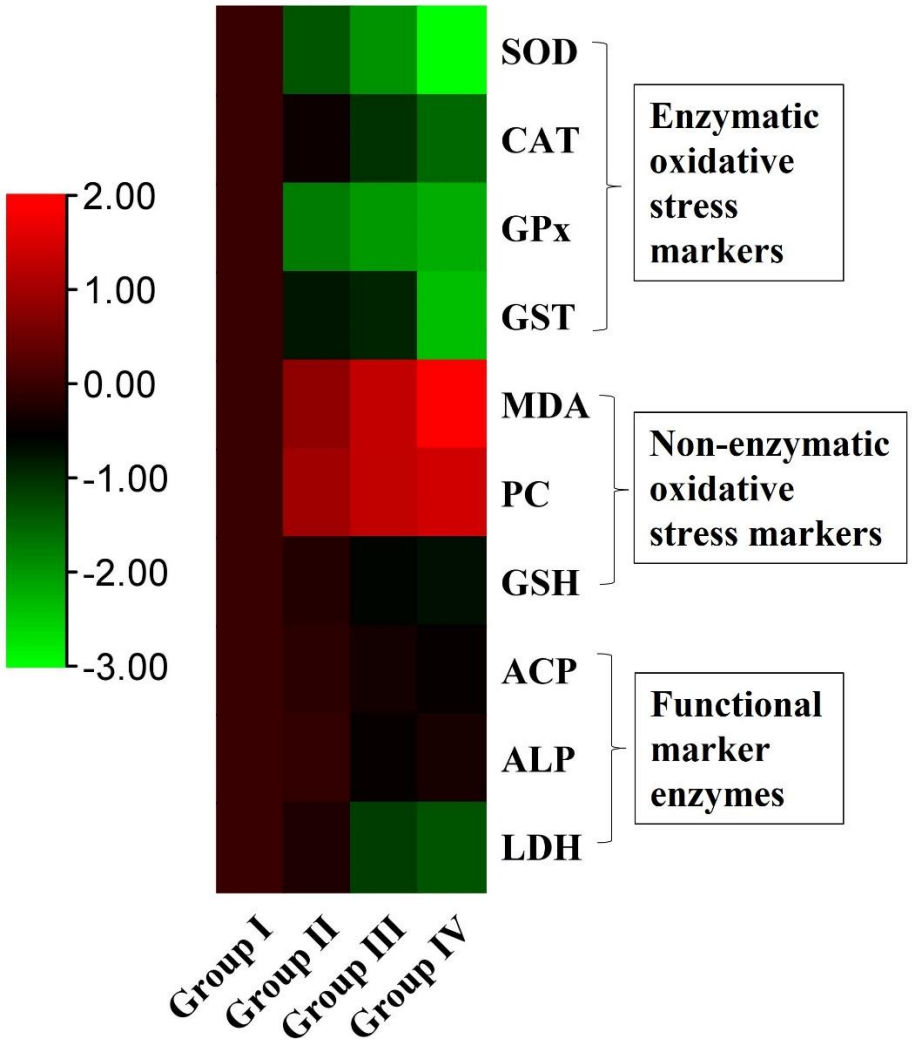


Figure 4: Heat map of respective parameters of all groups. The columns represent the selected parameters and the rows depict the different groups. Each cell is colorized based on the level of expression.

5.1.7 Testicular DNA fragmentation by administration of NaF

Testicular DNA status was evaluated by agarose gel electrophoresis (Figure 5). A single band of high molecular weight was present in all groups while formation of ladder due to generation of low molecular weight fragmented DNA was observed in group III and IV which indicated fluoride mediated DNA strand breakage.

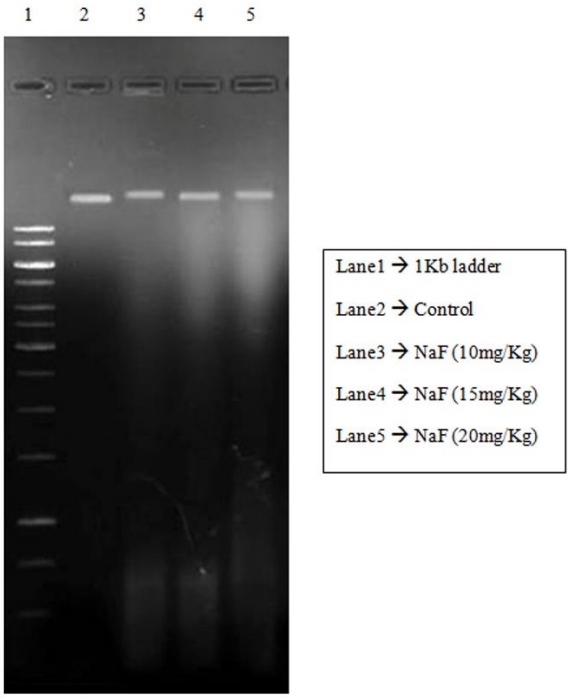


Figure 5: DNA fragmentation assay from isolated testicular genomic DNA of adult rats. Lane 1 represents 1 kb DNA ladder; Lane 2 represents only a single band of testicular genomic DNA of group I rats; Lane 3 represents a single band of testicular genomic DNA and slight trailing pattern of fragmented DNA of group II rats; Lane 4 and 5 represent single band with marked trailing of fragmented testicular genomic DNA of group III and IV rats respectively.

5.1.8 Altered spermatozoal DNA integrity by administration of NaF

Fluoride exposure resulted decreased number of green fluorescent spermatozoa having native double stranded DNA with a dose dependent manner (Figure 6).

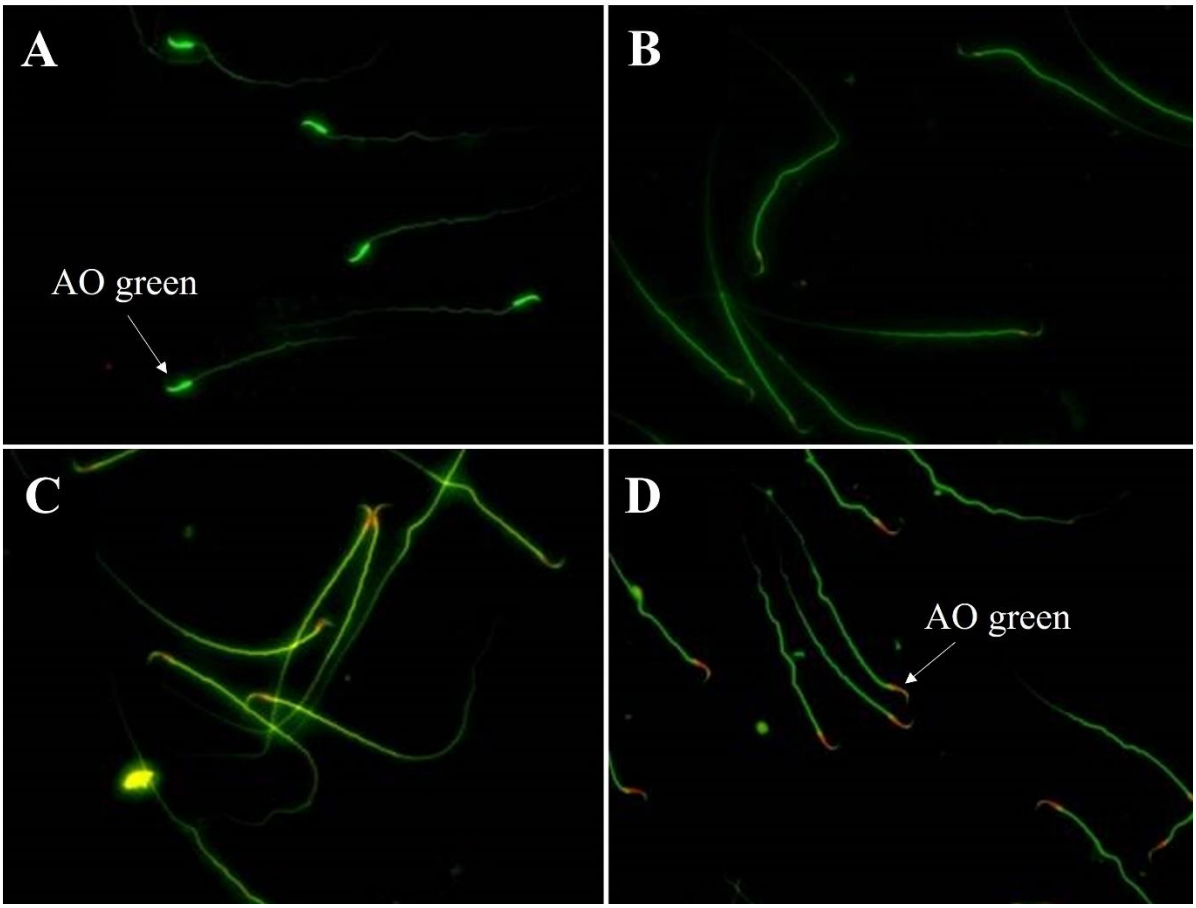


Figure 6: Photomicrograph of caudal spermatozoa stained with acridine orange (AO) at 400x magnification of group I (A), group II (B), group III (C) and group IV (D). AO green spermatozoa indicates native double stranded DNA of spermatozoa while AO red spermatozoa denotes damaged single stranded DNA.

5.2 Objective 2

This objective was dsigned to find out the efficacy of VC&VE against fluoride mediated testicular anomalies at the levels of structural entity, oxidative status, activities of functional parameters, steroidogenic status and DNA integrity. The results are presented here.

5.2.1 Role of VC&VE on fluoride induced altered body weight and gravimetry index

Body weight of rats was significantly ($p<0.01$) decreased in treated group while supplementation notably ($p<0.01$) recovered the body weight. A significant difference ($p<0.05$) was also observed between control and only VC&VE supplemented group. Application of NaF caused noticeable reduction of relative testicular weight ($p< 0.05$) while the weight of accessory reproductive organs remained unaltered. Supplementation of VC&VE restored the

testicular weight in spite of fluoride treatment ($p<0.05$). No noticeable change was observed between control and only VC&VE supplemented group in this regard (Table 7).

Table:7 Effect of VC&VE supplementation on body weight relative weight of primary and accessory reproductive organs in fluoride treated adult rats

Animals	Difference in body weight (final- initial) %	Gravimetric index (g %)			
		Testis	Epididymis	Prostate	Seminal vesicle
Group I	6.273±2.549	0.752±0.032	0.233±0.015	0.062±0.004	0.292±0.066
Group II	-7.898±3.417^{a**}	0.623±0.021^{a*}	0.216±0.008	0.051±0.003	0.207±0.023
Group III	10.036±2.246^{b**}	0.784±0.033^{b*}	0.264±0.011	0.063±0.007	0.301±0.015
Group IV	19.296±4.671^{a*}	0.837±0.040	0.258±0.014	0.062±0.006	0.273±0.014

Values are presented as mean ± SEM, n=8 and comparison is made by *Tukey's test*.
a = group I vs group II, III & IV; b = group II vs group III. *p< 0.05; ** p <0.01.

5.2.2 Effect of VC&VE on fluoride mediated testicular histology

Fluoride mediated distorted testicular structure was restored by supplementation with VC&VE (Figure 7). Fluoride caused degenerative changes of testis like disorganized seminiferous tubules, disarray of spermatogonial cells and reduced sperm concentration within seminiferous tubules and disintegrated Leydig cells. These deleterious effects were seen to be restored due to supplementation, and the only VC&VE supplementation exerted normal histological features.

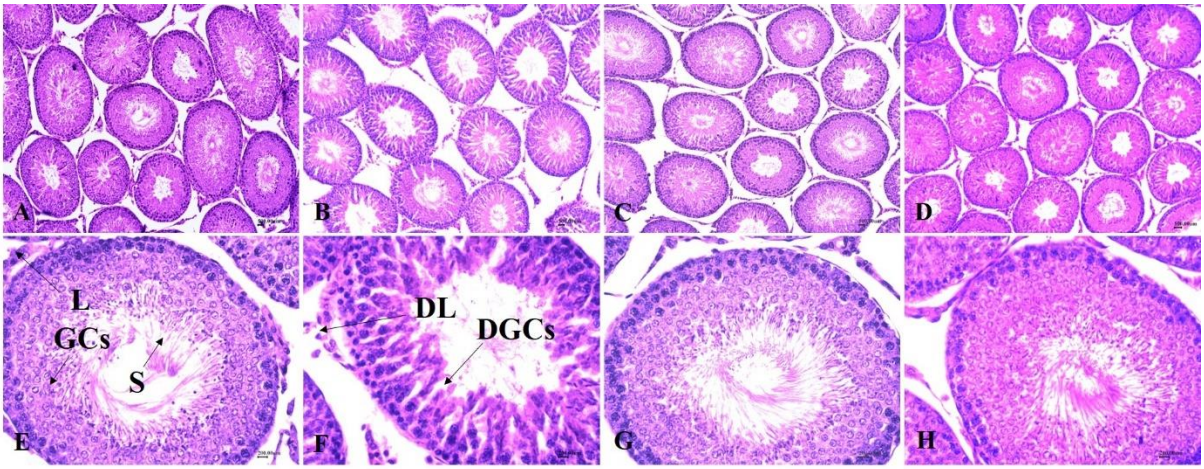


Figure7: Representative photomicrographs of H/E stained histoarchitecture of testes. A, B, C & D are testicular sections of group I, II, III & IV rats respectively at 100X magnification; whereas E, F, G & H represent same sections at 400X magnification. Germ cells (GCs), Leydig cells (L), spermatozoa (S), distorted germ cells (DGCs), disintegrated Leydig cells (DL).

5.2.3 Role of VC&VE on fluoride mediated altered testicular histomorphometry

Histomorphometric analysis revealed that fluoride caused significant decrease of seminiferous epithelial height ($p<0.001$), enlarged luminal diameter ($p<0.001$) and decreased Leydig cell nuclear area ($p<0.001$) while no noticeable change was observed in case of seminiferous tubule diameter. Supplementation resulted noteworthy recovery from all such damages ($p<0.01$ in case of seminiferous epithelial height; $p<0.001$ in case of luminal diameter and $p<0.001$ in case of Leydig cell nuclear area) while significant difference ($p<0.01$) still existed in case of Leydig cell nuclear area between group I and III. Only VC&VE supplementation exhibited all the features near normal level (Table 8, Figure 14).

Table:8 Effect of VC&VE supplementation on testicular histomorphometry of fluoride treated adult rats

Animals	STD (µm)	SEH (µm)	LD (µm)	LCNA (μm^2)
Group I	274.123±7.034	80.685±1.009	59.858±2.720	41.263±1.002
Group II	270.971±5.258	$70.940\pm0.960^{a***}$	$122.285\pm7.078^{a***}$	$29.586\pm0.908^{a***}$
Group III	266.745±1.353	$80.173\pm0.729^{b**}$	$55.343\pm1.335^{b***}$	$36.320\pm0.794^{a**b***}$
Group IV	266.721±4.050	81.936±1.042	60.951±2.534	40.988±0.294

Values are presented as mean ± SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III. ** $p<0.01$; ***$p<0.001$. STD- Seminiferous tubule diameter, LD- Lumen diameter, SEH- Seminiferous epithelial height, LCNA- Leydig Cell nuclear area.

5.2.4 Effect of VC&VE on fluoride induced altered ultra-structure of testis

Ultrastructure of testis from control rats obtained by scanning electron microscope revealed well organized seminiferous tubule, intact basement membrane, organized myoid cells and proper arrangement of spermatogenic cells in control rats. Fluoride caused partial degeneration of seminiferous tubules, disarray in basement membrane, distorted myoid cells and disorientation of spermatogenic cells. Such abnormalities were restored in supplemented group. Only VC&VE supplemented group exhibited normal features like control (Figure 8).

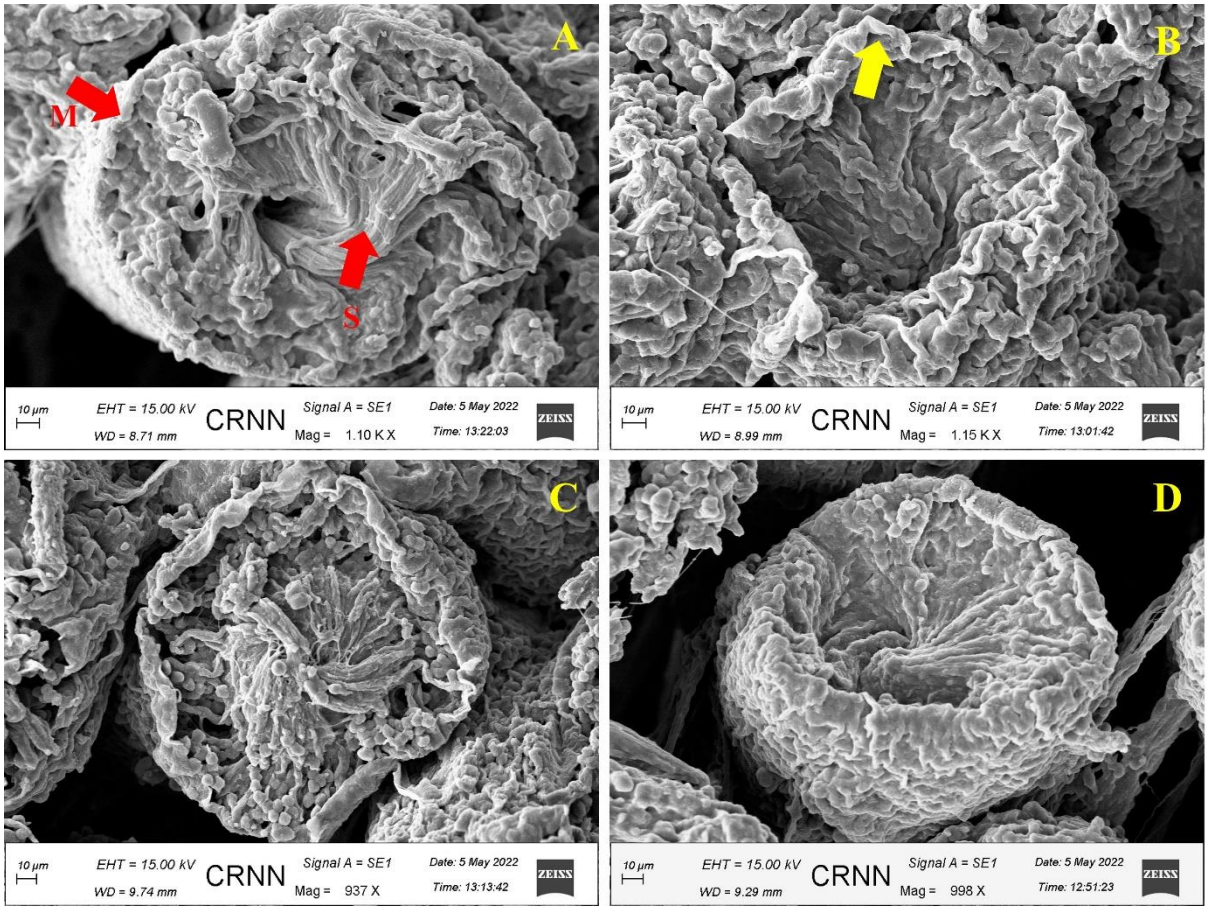


Figure 8: Representative photomicrographs of scanning electron microscopy of testes. A, B, C & D are testicular sections of group I, II, III & IV rats respectively. Myoid cells (M); Spermatozoa (S) and distorted myoid cells (yellow arrow).

5.2.5 Effect of VC&VE on fluoride mediated testicular oxidative stress

Exposure to fluoride altered the activities of enzymatic and non-enzymatic oxidative stress markers in testis (Table 9, Figure 14). Activities of enzymatic parameters like SOD, catalase, Gpx and GST reduced markedly ($p<0.001$ in all the cases) in fluoride treated rats. Level of GSH which is a non-enzymatic antioxidant marker was decreased notably ($p<0.001$) upon fluoride treatment. Significant restoration ($p<0.001$ in all cases) of the activities of such parameters was noted in supplemented group. Significant difference still existed between group I and III in case of SOD ($p<0.05$) and GPx ($p<0.001$) activities. Level of MDA and PC contents was enhanced suggestively ($p< 0.001$ in both the cases) in treated group. However, VC&VE supplementation restored the levels of MDA and PC in a significant way ($p<0.001$ in both cases). Only VC&VE supplementation did not exhibit any noticeable change with control.

Table 9: Effect of VC&VE on testicular oxidative stress in fluoride treated adult rats

Animals	Oxidative stress parameters						
	Enzymatic				Non enzymatic		
	SOD (unit/mg tissue)	Catalase (µM H_2O_2 consumed/ mg tissue/min)	GST (µM NADPH consumed/ min/mg protein)	GPx (nM NADPH oxidized/mg tissue/min)	MDA (nM/mg tissue)	PC (µM/mg protein)	GSH (µM/mg tissue)
Group I	3.524± 0.166	28.412±1. 458	716.953±3 .645	0.188±0.0 02	7.270±0.5 96	25.428±1.0 09	606.642±4. 680
Group II	1.002± 0.130^{a***}	12.335±1. 786^{a***}	494.525±6 6.715^{a***}	0.065±0.0 06^{a***}	15.507±0. 870^{a***}	51.836±2.9 64^{a***}	404.842±6. 228^{a***}
Group III	2.522± 0.150^{a*b***}	28.105±2. 025^{b***}	695.711±5 .793^{b***}	0.097±0.0 02^{a***b***}	7.99±0.53 5^{b***}	26.191±1.2 93^{b***}	593.210±4. 823^{b***}
Group IV	3.250±0.37 6	29.665±0. 584	716.425±4 .325	0.167±0.0 03	7.168±0.8 60	25.437±0.7 05	611.127±3. 689

Values are presented as mean ± SEM, n = 8 and comparison is made by *Tukey's test*.
a = group I vs group II, III & IV; b = group II vs group III. *p <0.05; ***p <0.001. SOD-Superoxide dismutase; GST-Glutathione S-transferase; GPx-Glutathione peroxides; MDA-Malondialdehyde; and PC-Protein carbonyl; GSH- Reduced glutathione.

5.2.6 Effect of VC&VE on relative protein expression testicular Nrf2 in fluoride treated rats

Expression of Nrf2 was significantly decreased (p<0.001) in fluoride treated rats which was evidenced by lower band intensity in treated group. Supplementation noteworthy restored (p<0.001) the level of Nrf2 in spite of fluoride treatment. No significant change in protein expression was observed between group I and IV (Figure 9).

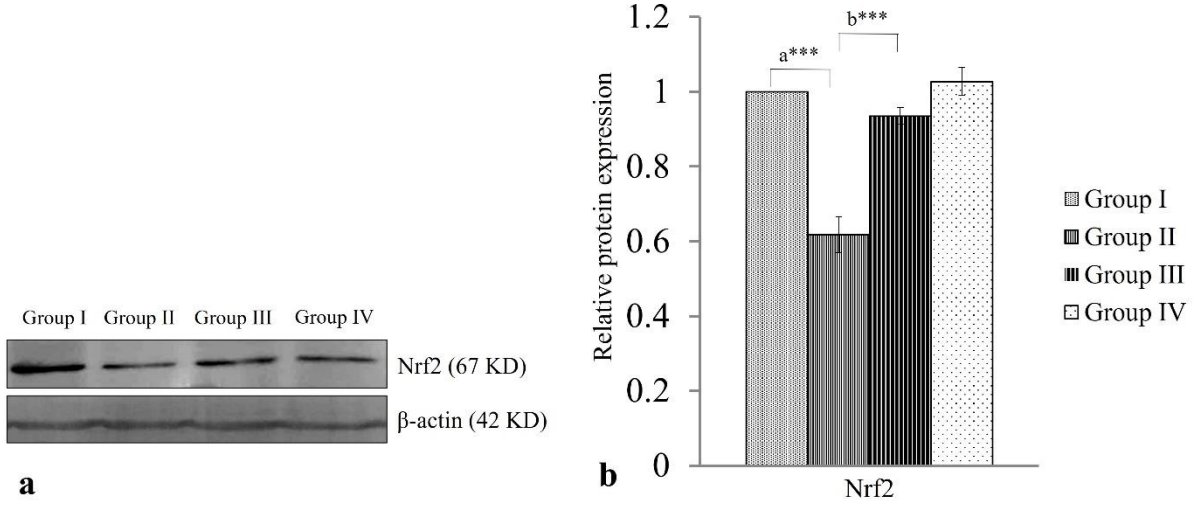


Figure 9: Testicular protein level expressions of Nrf2 was analyzed by Western Blotting and its densitometric analysis. β-actin was used as loading control and densitometric analysis was done with reference to corresponding band of β-actin expression. (a) indicates band intensities of respective groups; (b) indicates relative protein expression of Nrf2. Values are presented as

mean ± SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; ***p<0.001.

5.2.7 Role of VC&VE on fluoride mediated altered testicular functional activities

Noteworthy reduction of testicular functional status was observed in fluoride treated group whereas supplementation resulted noticeable recovery (Table 10, Figure 14). Activities of ACP, ALP and LDH were reduced remarkably in treated animals ($p < 0.001$ in all cases). Supplementation resulted significant recovery of activities of all the maker enzymes ($p < 0.01$ in case of ACP and $p < 0.001$ in case of ALP and LDH). Only VC&VE supplementation did not exert any noticeable difference with control group.

Table 10: Role of VC&VE on testicular functional marker enzymes in fluoride treated adult rats

Animals	Functional parameters of testis		
	ACP (µg of PNP liberated/ mg of tissue/ hr)	**ALP (µg of PNP liberated/ mg of tissue/ hr)**	**LDH (unit/mg protein)**
Group I	4.851±0.230	4.595±0.113	2.234±0.092
Group II	$3.230\pm0.412^{a***}$	$2.998\pm0.400^{a***}$	$0.990\pm0.057^{a***}$
Group III	$4.671\pm0.050^{b**}$	$4.742\pm0.051^{b***}$	$1.890\pm0.110^{b***}$
Group IV	4.649±0.043	4.727±0.012	2.203±0.084

Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. ACP-Acid phosphatase; ALP-Alkaline phosphatase; LDH-Lactate dehydrogenase. a = group I vs group II, III & IV; b = group II vs group III & IV. ** p <0.01; *** p <0.001.

5.2.8 Effect of VC&VE on fluoride induced altered steroidogenesis and serum gonadotropins

Fluoride caused notable reduction of testicular Δ^5, 3β-HSD and 17β-HSD activities ($p< 0.001$ in both cases) whereas supplementation resulted significant restoration of such activities ($p< 0.001$ in both the cases). Although serum FSH content was seen to be decreased meaningfully in fluoride treated group ($p< 0.001$) but the level of LH was not significantly altered. However, supplementation notably restored the level of FSH ($p<0.001$). Serum testosterone level was diminished remarkably ($p<0.001$) in fluoride treated group but supplementation also reinstated

such level significantly ($p<0.01$). Only VC&VE supplementation did not exhibit significant difference with control (Figure 10 and 14).

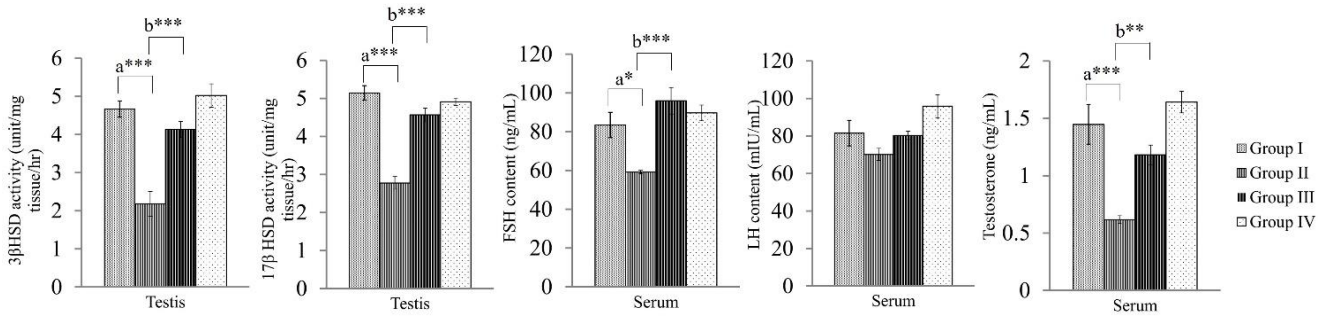


Figure 10: Effect of VC&VE on testicular steroidogenic status and gonadotropin level. Bar diagram depicts the amount of 3βHSD and 17βHSD in testicular tissue along with levels of serum FSH, LH and testosterone contents. Data is presented as mean ± SEM, n=8 and comparison is made by *Tukey's test.* a = group I vs group II, III & IV; b = group II vs group III. *p<0.05, **p<0.01 and *** p <0.001.

5.2.9 VC&VE preserve fluoride induced altered testicular DNA integrity

Testicular genomic DNA integrity was assessed by gel electrophoresis (Figure 11) where a single band of genomic DNA was appeared in all groups but a prominent smear of fragmented DNA was present in fluoride induced state. In supplemented group no such smear was present whereas the pattern was similar between group I and IV.

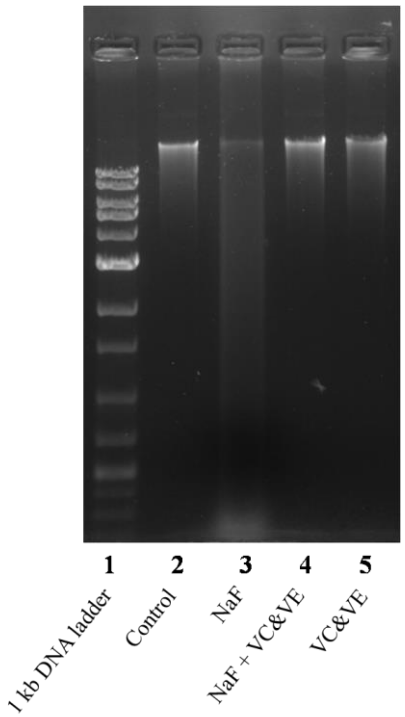


Figure 11: Assessment of testicular genomic DNA status by fragmentation assay of experimental rats. Lane 1 indicates 1 kb DNA ladder; Lane 2 represents a single band of genomic DNA of group I rats; Lane 3 represents trailing of fragmented genomic DNA along with a single band of group II rat; Lane 4 and 5 represent single band of genomic DNA of group III and IV rats.

5.2.10 VC&VE ameliorates fluoride induced cellular DNA damage of testis

Comet assay was performed to check cellular DNA status of testis which exhibited fluoride mediated testicular DNA damage as evidenced by increased comet tail length, tail moment and olive tail moment (Figure 12, 14; Table 11) in a significant way ($p < 0.001$). Supplementation caused notable recovery of such damages ($p < 0.001$) but not like control ($p < 0.001$). No significant difference was present between group I and IV.

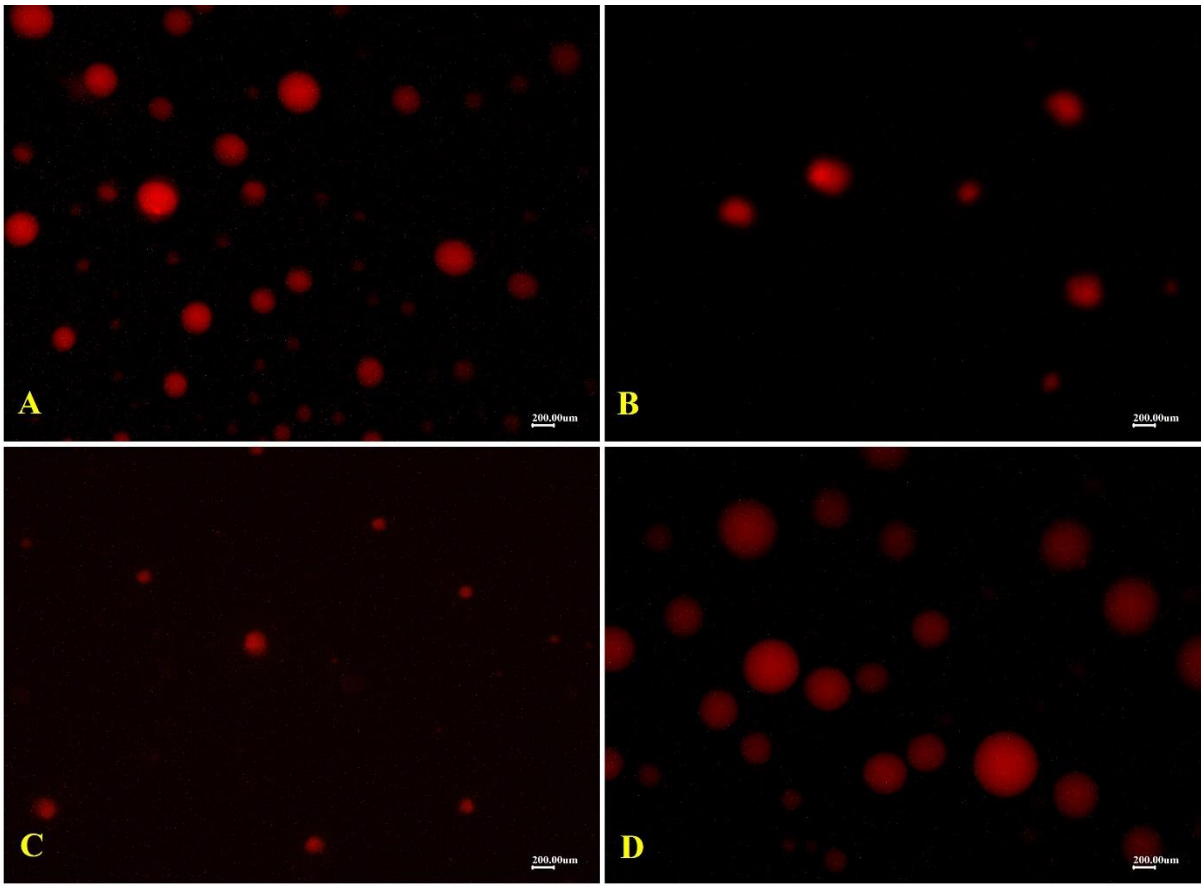


Figure 12: Photomicrographic representation of testicular comet assay in group I (A), group II (B), group III (C) and group IV (D) rats at 400 X magnifications.

Table 11: Effect of VC&VE supplementation on testicular DNA damage in fluoride treated adult rats

Parameters	Experimental rats			
	Group I	Group II	Group III	Group IV
Tail length	10.23±0.615	38.92±3.146^{a***}	22.28±0.777^{a***b***}	9.55±0.667^{b***}
Tail moment	9.12±0.750	37.17±1.985^{a***}	20.92±1.753^{a***b***}	7.66±0.968^{b***}
Olive moment	5.36±0.719	19.33±1.694^{a***}	10.71±0.695^{a***b***}	4.51±0.547^{b***}

Values are presented as mean ± SEM, n=8 and comparison is made by *Tukey's test*.
a = group I vs group II, III & IV; b = group II vs group III & IV. *** p <0.001.

5.2.11 Role of VC&VE on testicular fluoride accumulation

Fluoride content of testis was increased meaningfully in treated group (p<0.001). Supplementation caused marked reduction of the fluoride content (p< 0.001) but not at the level of control (p< 0.001). No variation was present between group I and IV (Figure 13).

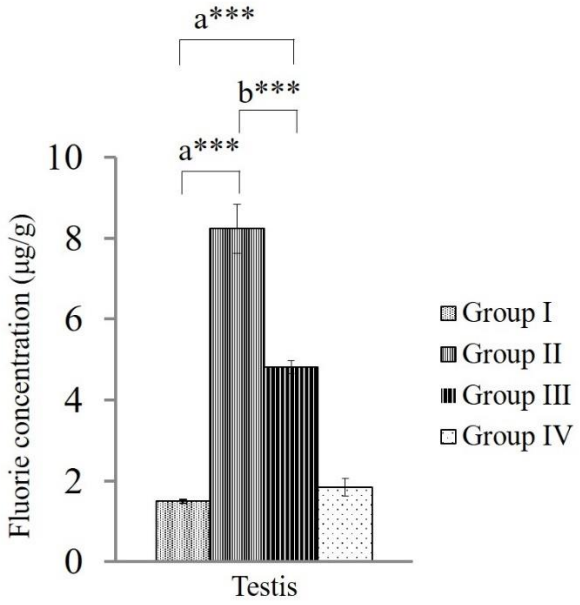


Figure 13: Effect of VC&VE on testicular fluoride content. Bar diagram depicts the amount of accumulated fluoride in the respective tissues. Data is presented as mean±SEM, n=8 and comparison is made by *Tukey's test.* a = group I vs group II, III & IV; b = group II vs group III & IV. *** p <0.001.

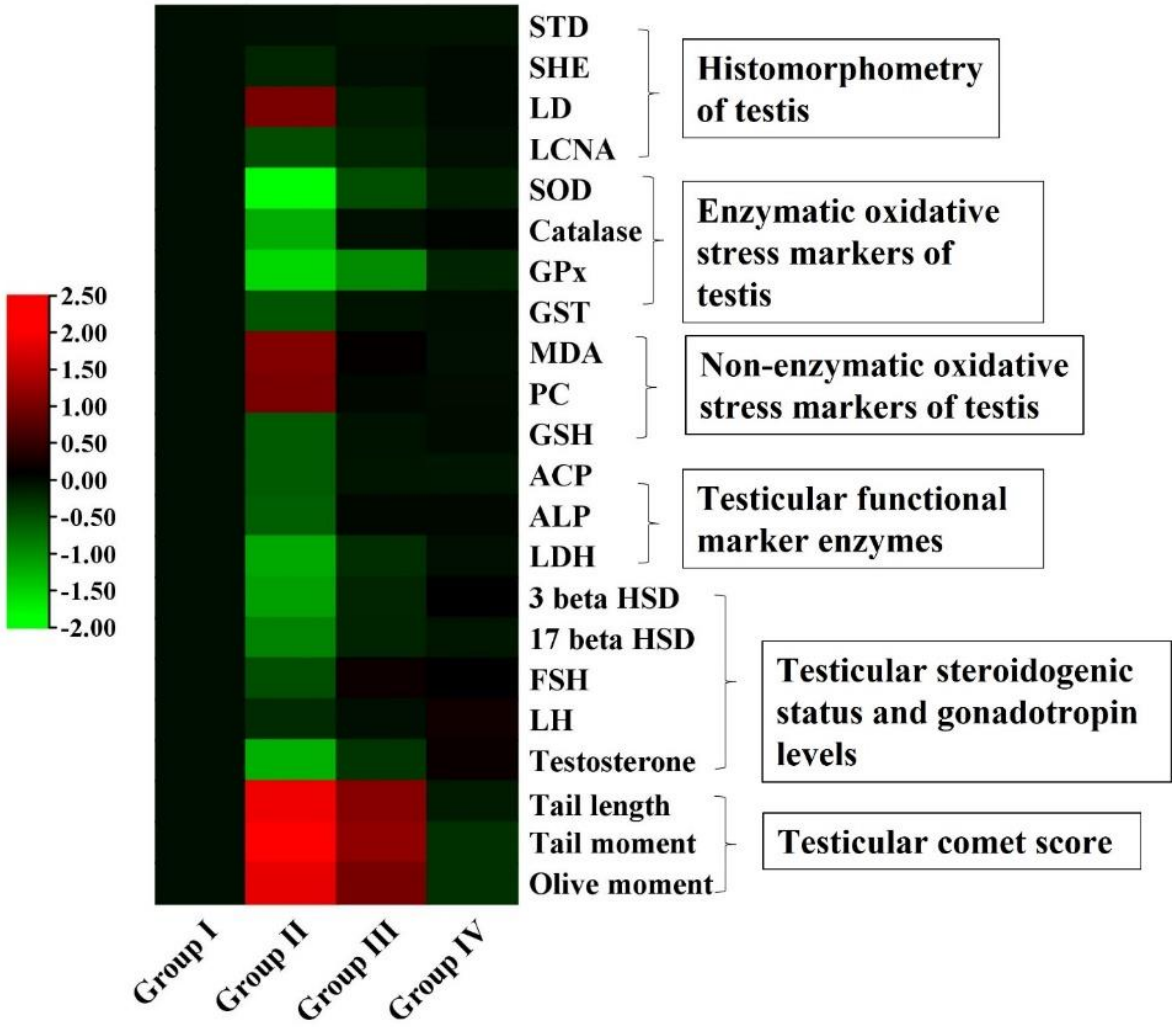


Figure 14: Heat map of respective parameters of all groups. The columns represent the selected parameters and the rows depict the different groups. Each cell is colorized based on the level of expression.

5.3 Objective 3

This objective was framed to find out the efficacy of VC&VE against fluoride mediated epididymal and spermatozoal anomalies at the levels of structural entity, redox status, spermatozoal potential and DNA integrity. The results are presented here.

5.3.1 Effects of VC&VE on epididymal histology

Histological features of both caput and cauda epididymis from control rats has been characterized by the presence of lobules lined by pseudostratified epithelium having stereocilia

on the luminal surface and presence of dense spermatozoal population within lumens. Fluoride treatment did not show any noticeable alteration/s in gross structure of caput region (Figure 15) but marked structural degeneration of cauda region was observed in terms of thinning of epithelial layer, decreased luminal sperm concentration and less appearance of stereocilia (Figure 16). Supplementation caused noticeable restorations of such structural anomalies of cauda. Only VC&VE supplementation exhibited same structural organization like control.

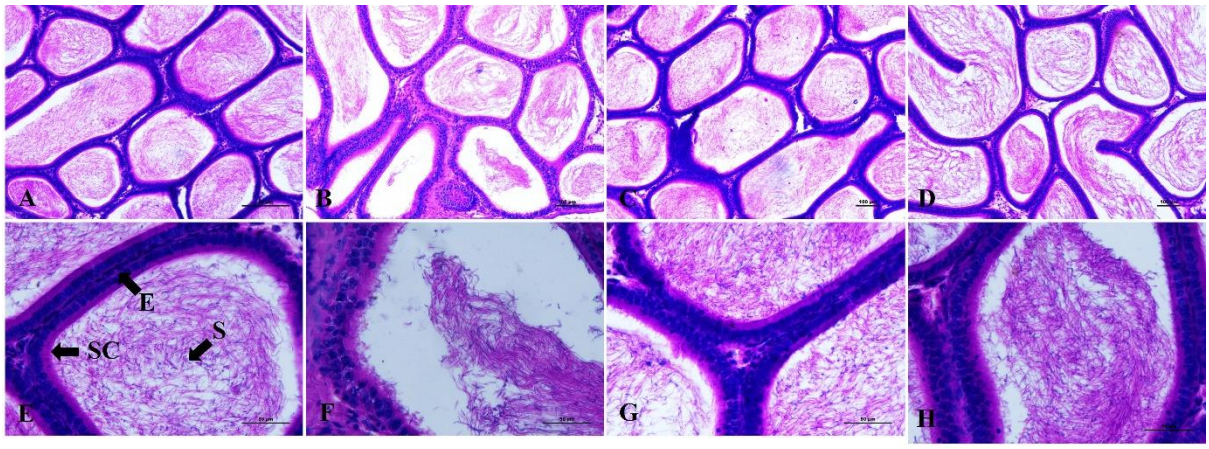


Figure 15: Representative images of epididymal (caput) histoarchitecture evaluated by H/E staining. A & E are representative tissue sections at 100X and 400X magnification of group I rats; B & F are tissue sections at 100X and 400X magnification of group II rats; C & G are tissue sections at 100X and 400X magnification of group III rats; D & H are tissue sections at 100X and 400X magnification of group IV rats. Stereocilia (SC), Epithelial lining (E), spermatozoa (S).

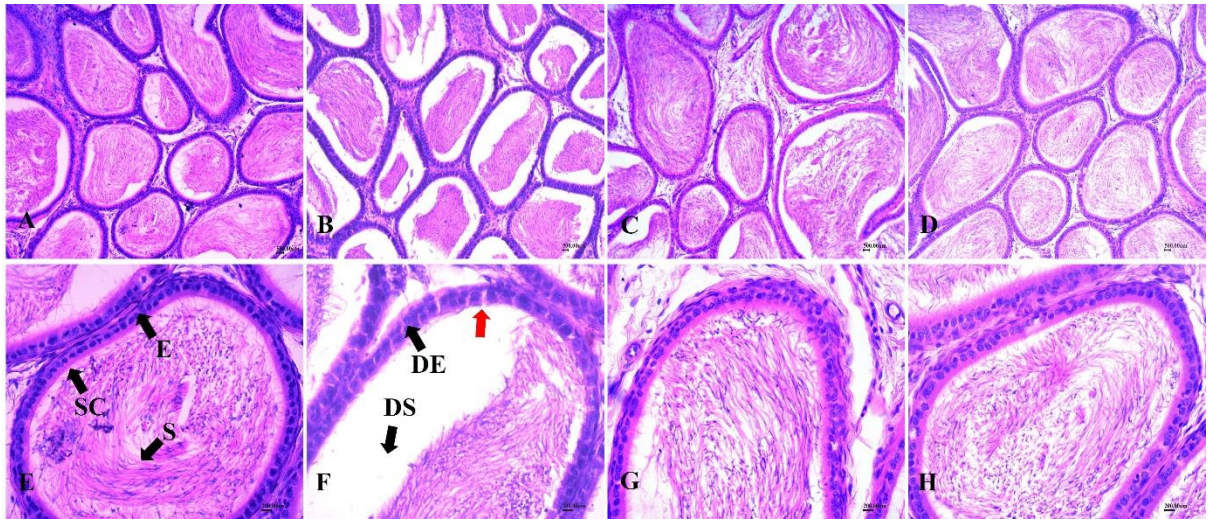


Figure 16: Representative images of epididymal (cauda) histoarchitecture evaluated by H/E staining. A & E are representative tissue sections at 100X and 400X magnification of group I rats; B & F are tissue sections at 100X and 400X magnification of group II rats; C & G are tissue sections at 100X and 400X magnification of group III rats; D & H are tissue sections at 100X and 400X magnification of group IV rats. Stereocilia (SC), Epithelial lining (E),

spermatozoa (S), distorted epithelium (DE), decreased sperm concentration (DS), reduced stereocilia (red arrow).

5.3.2 Role of VC&VE on ultra-structure of epididymis

Ultra-structural analysis by SEM clearly revealed that fluoride did not cause any noticeable change of caput epididymis (Figure 17) while decreased spermatozoal concentration was observed in cauda. Supplementation exhibited marked recovery from such altered structural entity in case of cauda epididymis (Figure 18). Only VC&VE supplemented groups exhibited similar structural organization in both caput and cauda.

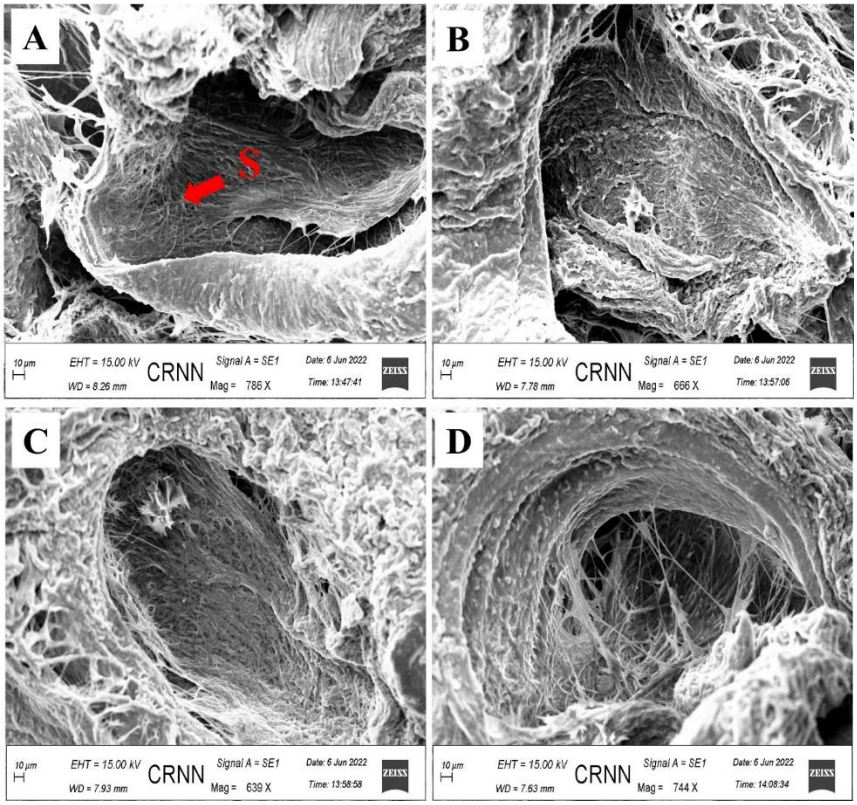


Figure 17: Representative photomicrographs of scanning electron microscopy of caput. A, B, C & D are the sections of group I, II, III & IV rats respectively. Spermatozoa (S).

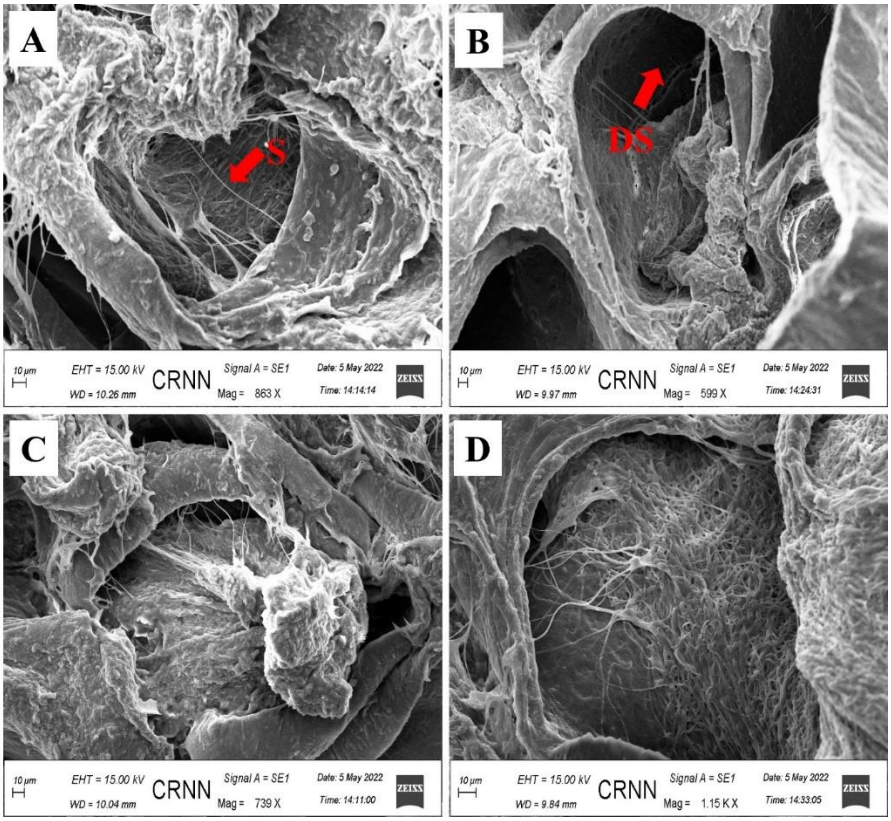

A
S
10 µm EHT = 15.00 kV WD = 10.26 mm CRNN Signal A = SE1 Mag = 863 X Date: 5 May 2022 Time: 14:14:14 ZEISS
B
DS
10 µm EHT = 15.00 kV WD = 9.97 mm CRNN Signal A = SE1 Mag = 599 X Date: 5 May 2022 Time: 14:24:31 ZEISS
C
10 µm EHT = 15.00 kV WD = 10.04 mm CRNN Signal A = SE1 Mag = 739 X Date: 5 May 2022 Time: 14:11:00 ZEISS
D
10 µm EHT = 15.00 kV WD = 9.84 mm CRNN Signal A = SE1 Mag = 1.15 K X Date: 5 May 2022 Time: 14:33:05 ZEISS

Figure 18: Representative photomicrographs of scanning electron microscopy of cauda. A, B, C & D are the sections of group I, II, III & IV rats respectively. Spermatozoa (S), decreased luminal spermatozoa (DS).

5.3.3 Effect of VC&VE on epididymal oxidative stress

No marked alteration in the activities of SOD and GSH as well as no significant changes of MDA and PC contents were observed in caput region of epididymis after fluoride threat, while catalase activity was notably decreased in treated rats ($p<0.05$). Supplementation exerted noticeable recovery ($p<0.05$) in case of catalase activity. (Table 12).

Activities of SOD ($p<0.01$) and catalase ($p<0.001$) along with the GSH content ($p<0.001$) in the cauda region of epididymis were significantly decreased in fluoride treated rats. Noticeable augmentation of MDA and PC contents were also observed ($p<0.001$ in both cases). However, supplementation exerted conspicuous recovery of SOD ($p<0.01$) and catalase ($p<0.001$) activities as well GSH level ($p<0.001$). Significant restoration of MDA ($p<0.01$) and PC ($p<0.001$) contents were also noted upon supplementation (Table 13).

There was no significant change in the expression of all markers between group I and IV.

Table 12: Effect of VC&VE on epididymal (caput) oxidative stress in fluoride treated adult rats

Animals	Oxidative stress parameters				
	Enzymatic		Non enzymatic		
	SOD (unit/mg tissue)	Catalase (µM H_2O_2 consumed/mg tissue/min)	MDA (nM/mg tissue)	PC (µM/mg protein)	GSH (µM/mg tissue)
Group I	2.9333±0.281	21.7317±0.490	3.4367±0.355	10.3733±0.288	193.91±11.702
Group II	2.2167±0.170	19.6000±0.335^{a*}	4.0783±0.322	11.5567±0.449	168.95±8.073
Group III	2.9833±0.2700	21.2417±0.634^{b*}	3.3067±0.235	10.6750±0.369	188.55±6.903
Group IV	3.3667±0.230	22.0483±0.353	2.9833±0.242	10.2350±0.291	201.84±11.761

Values are presented as mean ± SEM, n = 8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III *p <0.05. SOD-Superoxide dismutase; MDA- Malondialdehyde; and PC-Protein carbonyl; GSH- Reduced glutathione.

Table 13: Effect of VC&VE on epididymal (cauda) oxidative stress in fluoride treated adult rats

Animals	Oxidative stress parameters				
	Enzymatic		Non enzymatic		
	SOD (unit/mg tissue)	Catalase (µM H_2O_2 consumed/mg tissue/min)	MDA (nM/mg tissue)	PC (µM/mg protein)	GSH (µM/mg tissue)
Group I	2.3748±0.5166	24.7517±0.33991	4.3233±1.0568	12.0817±0.1012	206.3300±3.0010
Group II	0.6058±0.1101^{a**}	14.5000±1.1222^{a***}	11.7117±0.5642^{a***}	24.3200±1.2077^{a***}	156.9767±1.7960^{a***}
Group III	1.9397±0.1401^{b**}	23.8450±0.7099^{b***}	4.6917±0.5793^{b***}	13.7867±0.8348^{b***}	203.2600±2.1052^{b***}
Group IV	2.1633±0.1689	24.5850±0.3349	3.6767±0.4450	12.6533±0.2333	212.3533±2.4888

Values are presented as mean ± SEM, n = 8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III **p <0.01; ***p<0.001. SOD-Superoxide dismutase; MDA- Malondialdehyde; and PC-Protein carbonyl; GSH- Reduced glutathione.

5.3.4 Effect of VC&VE on relative expression of epididymal (cauda) Nrf2 in fluoride exposed rats

Immunoblotting analysis from cauda region of epididymis had shown that the relative expression of Nrf2 was decreased significantly (p<0.001) due to fluoride treatment. Supplementation augmented such level (p<0.001) in spite of fluoride treatment. Only VC&VE supplementation did not exhibit any change in the protein expression compared to that of control (Figure 19).

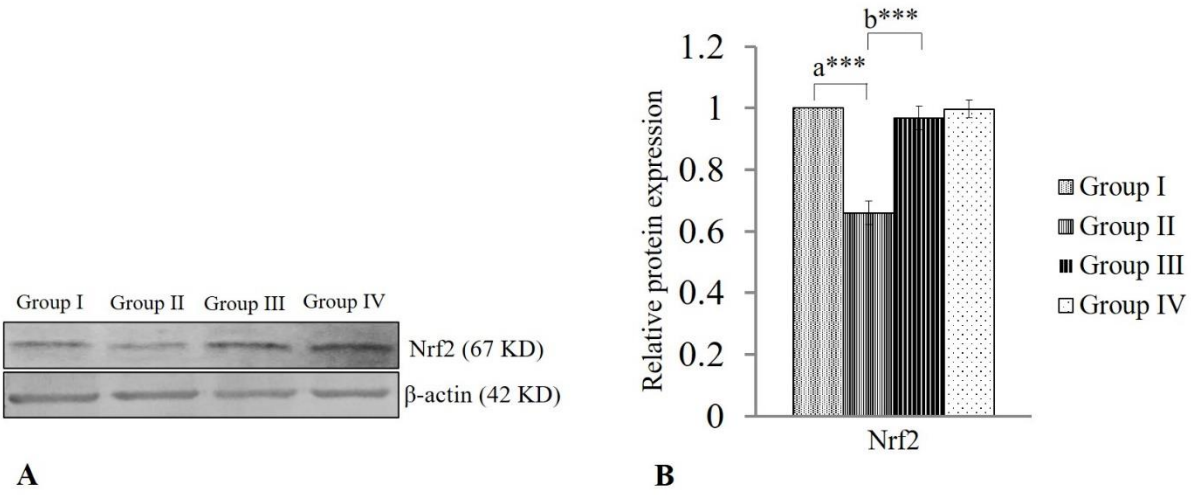


Figure 19: Epididymal (cauda) protein level expressions of Nrf2 were analyzed by Western Blotting and its densitometric analysis was presented. β-actin was used as loading control and densitometric analysis was done with reference to the corresponding band of β-actin expression. (A) indicates band intensities of respective groups; (B) indicates relative protein expression of Nrf2. Values are presented as mean ± SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; ***p<0.001.

5.3.5 Effect of VC&VE on epididymal DNA damage in fluoride treated rats

Epididymal genomic DNA integrity was analysed by agarose gel electrophoresis. In caput epididymis only a band of genomic DNA was prominent in all groups (Figure 20 A). No smear of fragmented DNA was observed in treated animals.

In case of cauda epididymis, a single band of genomic DNA was observed in all groups but a prominent smear of fragmented DNA was appeared in fluoride treated group. In case of supplemented animals, no such smear was observed. The pattern of genomic DNA was similar between group I and IV (Figure 20 B).

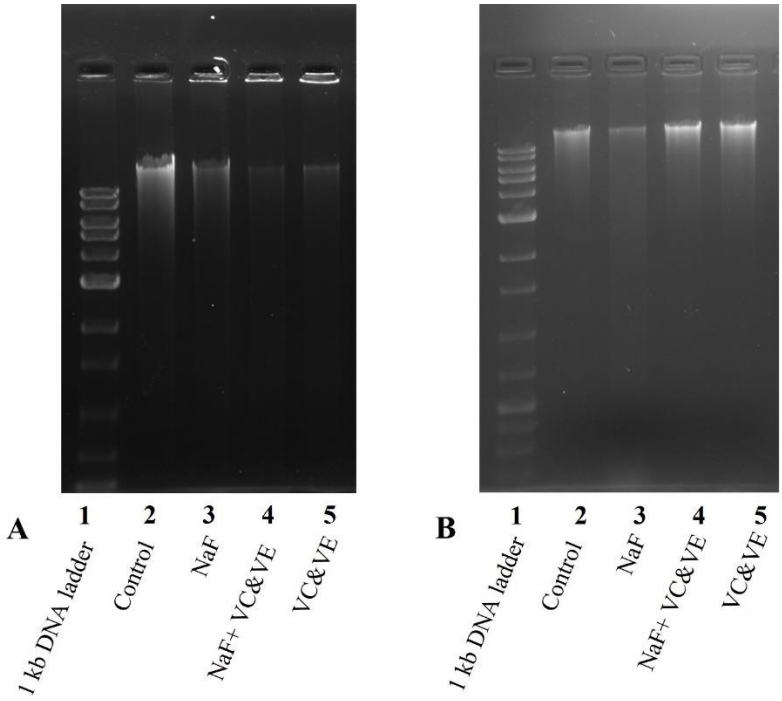


Figure 20: DNA fragmentation assay from isolated genomic DNA from caput (A) and cauda (B) regions of epididymis of adult rats. Lane 1 represents 1 kb DNA ladder; Lane 2, 3, 4 and 5 represent genomic DNA of group I, II, III and IV rats.

5.3.6 Role of VC&VE on spermatozoal morphology in fluoride treated adult rats

Eosin/Nigrosine staining was also used to evaluate the structural anomalies of spermatozoa which have been documented in figure 21 (Table 14). Exposure to fluoride significantly increased the percentage of both primary and secondary abnormalities of spermatozoa. Supplementation restored/ prevented these changes significantly. No difference was present between group I and IV.

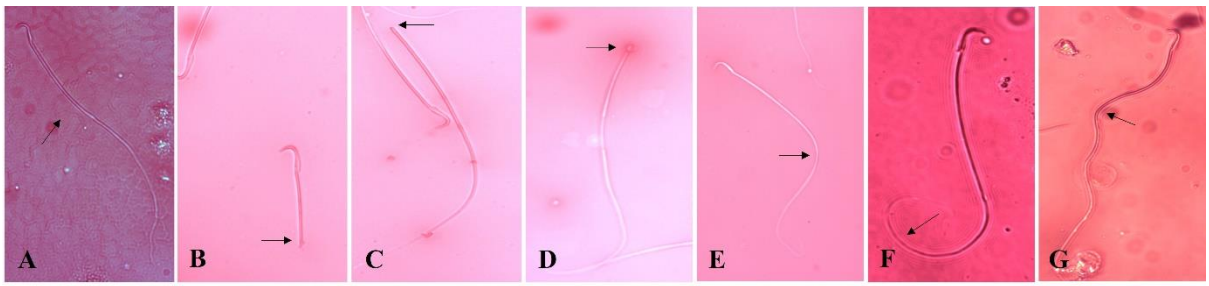


Figure 21: Eosin-nigrosin stained images of epididymal spermatozoa with structural anomalies. Normal spermatozoa (A), rudimentary tail (B), headless spermatozoa (C), pin head spermatozoa (D), curved tail (E), coiled tail (F) and curved midpiece (G).

Table 14: Effect of VC&VE supplementation on structural anomalies of caudal spermatozoa under fluoride exposed state epididymis in fluoride treated adult rats

Animals	Structural abnormalities (Morphology %)					
	Primary	Secondary				
	RT	HT	PT	CUT	COT	CMT
Group I	3.32±0.184	4.44±0.376	3.75±0.160	4.58±0.158	3.26±0.403	3.17±0.505
Group II	6.74±0.713^{a***}	7.72±0.442^{a***}	6.1825±0.334^{a***}	6.58±0.614^{a**}	6.92±0.173^{a***}	6.65±0.505^{a***}
Group III	3.58±0.250^{b***}	4.24±0.280^{b***}	3.64±0.201^{b***}	3.75±0.197^{b***}	3.64±0.185^{b***}	3.86±0.104^{b***}
Group IV	3.02±0.137	3.49±0.137	3.59±0.111	3.38±0.115	3.14±0.072	3.27±0.062

Values are presented as mean ± SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; **p<0.01; ***p<0.001. RT- rudimentary tail, HT- headless tail, PT- pin head tail, CUT- curve tail, COT- coiled tail and CMP- curved mid piece.

5.3.7 Effect of VC&VE on ultrastructure of spermatozoa and head structure in fluoride treated adult rats

Photomicrographic presentation by SEM also divulged the extent of spermatozoal structural anomalies in fluoride treated rats (Figure 22). Although theses anomalies of spermatozoa were present in all groups but the occurrence was higher extent in group II rats as compared to others. Ultra-structure of spermatozoal head of control rats showed normal features like intact perforatium (sub-acrosomal material), intact plasma membrane, nucleus, post nuclear cap and post acrosomal dense laminar region (Figure 23 A). Bulged perforatium, distorted post nuclear cap and post acrosomal dense laminar region were observed in fluoride treated rats (Figure 23 B). In supplemented and only VC&VE supplemented animals the features of spermatozoal head were like those of control (Figure 23 C & D).

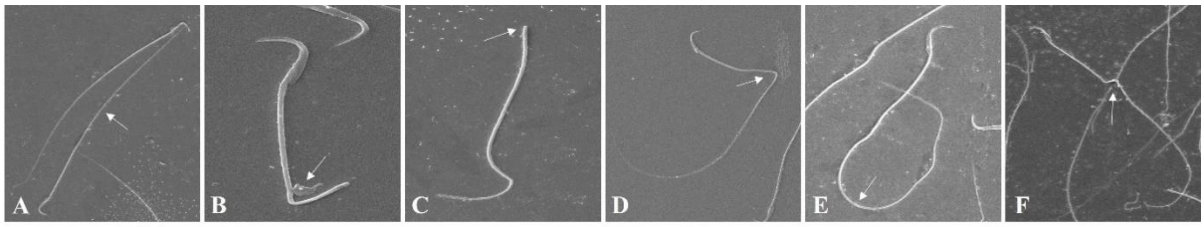
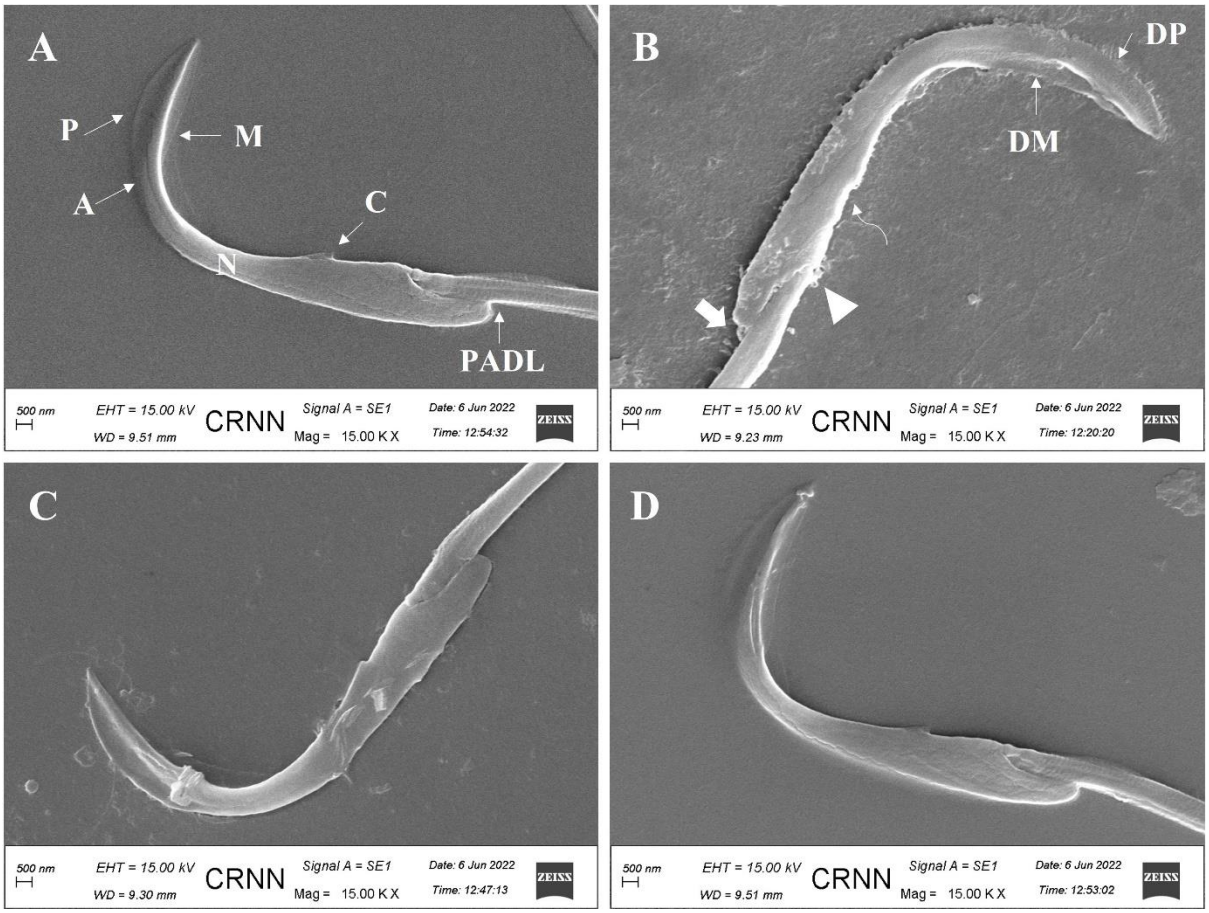


Figure 22: Scanning electron micrographs of epididymal spermatozoa. Normal spermatozoa (A), rudimentary tail (B), headless tail (C), curved tail (D), coiled tail (E), curved midpiece (F) were the observed spermatozoal morphology.


A
P
M
A
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PADL
500 nm EHT = 15.00 kV WD = 9.51 mm CRNN Signal A = SE1 Mag = 15.00 K X Date: 6 Jun 2022 Time: 12:54:32 ZEISS
B
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500 nm EHT = 15.00 kV WD = 9.23 mm CRNN Signal A = SE1 Mag = 15.00 K X Date: 6 Jun 2022 Time: 12:20:20 ZEISS
C
500 nm EHT = 15.00 kV WD = 9.30 mm CRNN Signal A = SE1 Mag = 15.00 K X Date: 6 Jun 2022 Time: 12:47:13 ZEISS
D
500 nm EHT = 15.00 kV WD = 9.51 mm CRNN Signal A = SE1 Mag = 15.00 K X Date: 6 Jun 2022 Time: 12:53:02 ZEISS

Figure 23: Scanning electron microscopic study of spermatozoal head from group I (A), group II (B), group III (C) and group IV (D) rats. Spermatozoa of control rat (A) exhibits normal features like intact acrosome (A); post nuclear cap (C); plasma membrane (M); nucleus (N); perforatorium (subacrosomal material) (P) and post acrosomal dense laminar region (PADL). Spermatozoa from treated rat (B) depicts several anomalies like distorted perforatorium (DP), distorted plasma membrane (DM), inaccurate post nuclear cap (curved arrow), distorted PADL (large arrow) and serration at connective piece of spermatozoa (arrow head). Spermatozoa of supplemented (C) and only supplemented (D) rats displayed nearly normal structure to that of control rat.

5.3.8 Effect of VC&VE on sperm potential in fluoride treated rats

Substantial reduction of count ($p<0.001$), motility ($p<0.001$) and the percentage of HOS+ve spermatozoa ($p<0.01$) were observed due to fluoride treatment along with no noticeable decreased of viability were obtained when they were collected from the caput (Table 15). Supplementation exhibited noteworthy recovery of number ($p<0.001$) and motility ($p<0.01$) while percentage of HOS+ve spermatozoa was not restored significantly.

No marked alteration was observed between group I and IV in case of all parameters.

All studied parameters related to sperm potential, when isolated from cauda, were altered ($p<0.001$ in all the cases) in fluoride treated rats (Table 16). Supplementation resulted noteworthy restoration all the features like number ($p<0.05$), motility ($p<0.001$), viability ($p<0.001$) and hypo-osmotic swelling ($p<0.001$).

No marked alteration was observed between group I and IV in case of all parameters.

Table 15: Effect of VC&VE on sperm potentials isolated from caput epididymis of fluoride induced rats

Animals	Sperm parameters			
	Count (x 10^6)	Motility (%)	Viability (%)	HOS +ve (%)
Group I	80.99 ± 2.088	79.50 ± 2.202	75.33 ± 0.714	76.50 ± 1.176
Group II	66.91 ± 0.733^{a***}	64.50 ± 1.204^{a***}	68.83 ± 1.887	68.50 ± 1.431^{a**}
Group III	78.62± 1.858^{b***}	76.16 ± 2.400^{b**}	73.83 ± 3.134	72.50 ± 1.765
Group IV	85.78 ± 0.921	84.66 ± 2.060	72.33 ± 0.557	75.50 ± 1.310

Values are presented as mean ± SEM, n=8 and comparison is made by *Tukey's test*. HOS: Hypo-osmotic swelling. a = group I vs group II, III & IV; b = group II vs group III & IV. **p< 0.01; *** p <0.001.

Table 16: Effect of VC&VE on sperm potentials isolated from cauda epididymis of fluoride treated rats

Animals	Sperm parameters			
	Count (x 10^6)	Motility (%)	Viability (%)	HOS +ve (%)
Group I	107.90 ± 2.419	94.83 ± 1.013	82.83 ± 1.013	83.33 ± 1.686
Group II	81.85 ± 2.560^{a***}	54.33 ± 1.282^{a***}	54.00 ± 1.366 ^{a***}	49.33 ± 1.115^{a***}
Group III	94.27 ± 4.599^{b*}	92.83 ± 1.301^{b***}	77.83 ± 1.922^{b***}	78.66 ± 1.021^{b***}
Group IV	101.65 ± 4.675	91.50 ± 0.846	79.33 ± 0.714	79.83 ± 1.470

Values are presented as mean ± SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III & IV. *p< 0.05; *** p <0.001. HOS: Hypo-osmotic swelling.

5.3.9 Effect of VC&VE on spermatozoal ROS generation in fluoride treated rats

Fluoride significantly enhanced spermatozoal ROS generation both in caput ($p<0.01$) and cauda ($p<0.001$). Supplementation exhibited notable recovery from augmented ROS generation in both caput ($p<0.05$) and cauda ($p<0.001$). There was no noticeable change between group I and IV (Figure 24, 25 and 26).

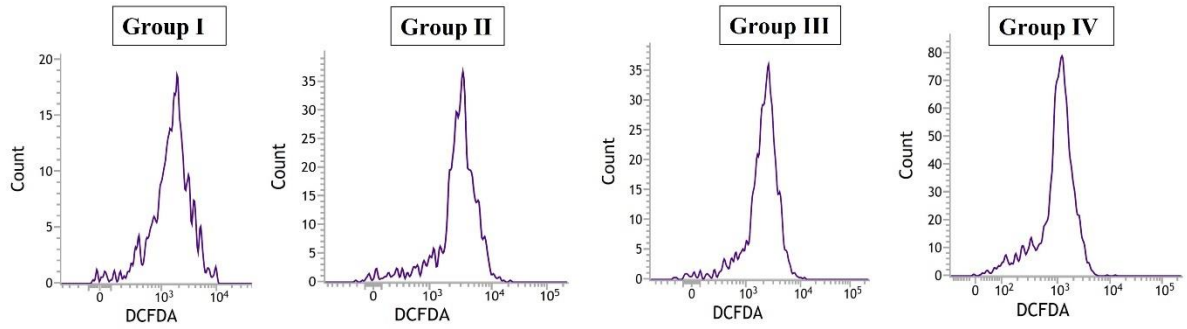

Figure 24: Effect of VC&VE on fluoride induced ROS generation of spermatozoa isolated from caput region of epididymis.

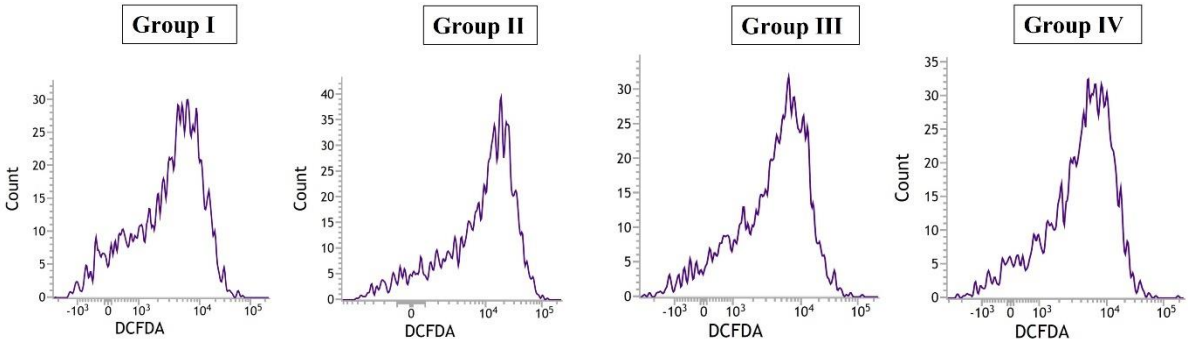

Figure 25: Effect of VC&VE on fluoride induced augmented ROS generation in caudal spermatozoa.

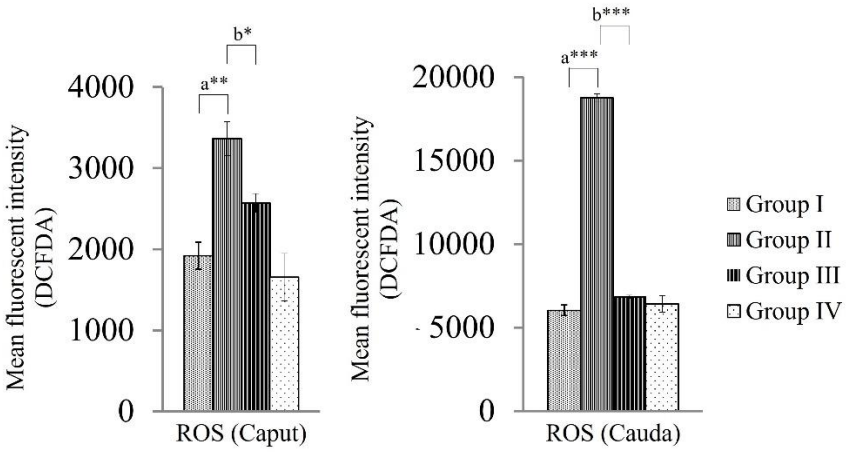

Figure 26: Effect of VC&VE on fluoride induced augmented spermatozoal ROS production by flow cytometric analysis using DCFDA. Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; *p<0.05, **p<0.01, ***p<0.001.

5.3.10 Role of VC&VE on fluoride mediated spermatozoal maturity and DNA integrity

Fluoride treatment increased histone rich immature spermatozoa in both caput ($p<0.001$) and cauda ($p<0.001$) which was evidenced by higher percentage of AB+ve spermatozoa as compared to that of control. Altered percentage was seen to minimize ($p<0.001$) after supplementation with VC&VE both in caput and cauda though considerable differences were still present ($p<0.001$ in both the cases) with control (Figure 27).

Spermatozoal DNA integrity which was assessed by AO staining exhibited similar pattern (Figure 28). Treatment with NaF caused significant decrease of the number of spermatozoa having native DNA as evidenced by reduced percentage of green fluorescent spermatozoa. This was due to meta-chromatic shifting and observed in spermatozoa isolated from both caput and cauda epididymis ($p<0.001$ in both the cases). However, supplementation resulted significant recovery ($p<0.001$ in both the cases) but not at the level of control ($p<0.001$ in both the cases).

No noticeable difference was present between group I and IV in case of both caput and cauda.

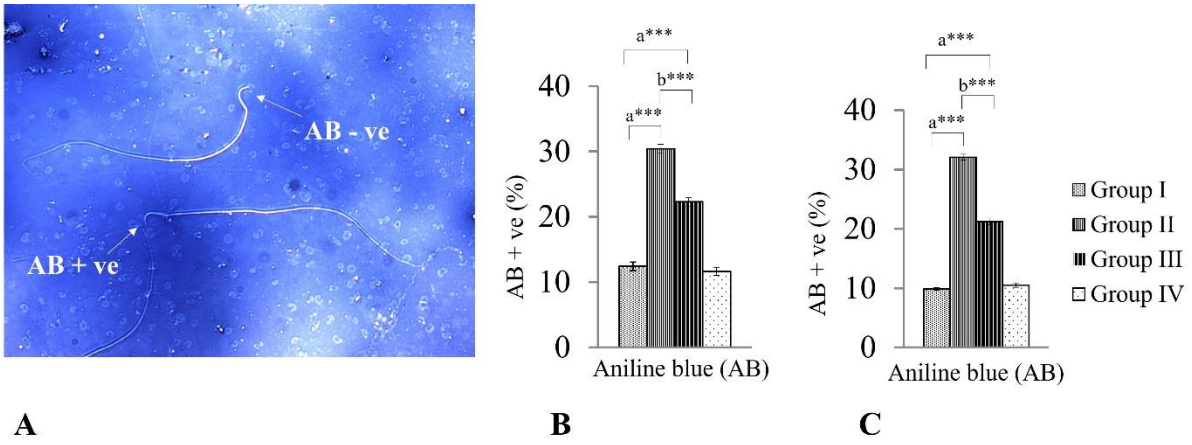

Figure 27: (A) Photomicrograph of caudal spermatozoa stained with aniline blue (AB) at 400X magnification. Spermatozoa with deep blue head (AB+ve) indicates immature spermatozoa whereas colourless head of spermatozoa (AB-ve) indicates mature spermatozoa; (B & C) Effects of VC&VE on fluoride mediated altered spermatozoal maturity isolated from caput and cauda respectively. Bar diagram depicts the number of AB+ve spermatozoa. Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; ***$p<0.001$.

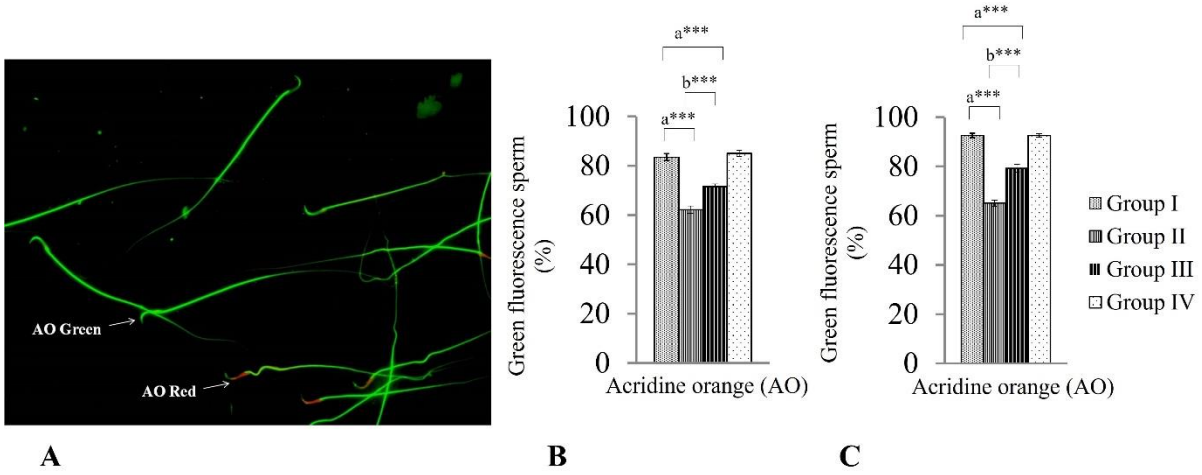

Figure 28: (A) Photomicrograph of caudal spermatozoa stained with acridine orange (AO) at 400X magnification. AO green spermatozoa indicates native double stranded DNA of spermatozoa while AO red spermatozoa denotes damaged single stranded DNA; (B & C) Effects of VC&VE on fluoride mediated altered spermatozoal DNA integrity isolated from caput and cauda respectively. Bar diagram depicts the percentage of AO green spermatozoa. Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; ***p<0.001.

5.4 Objective 4:

This objective was framed to find out the efficacy of VC&VE against fluoride mediated altered signaling cascades in testis and epididymis as well as spermatozoal apoptosis.

5.4.1 Role of VC&VE on testicular mRNA expressions of Bax, Bcl2, Bad and Bak genes in fluoride treated rats

The RTPCR data firmly divulged that fluoride significantly increased the expressions of pro-apoptotic genes such as Bax, Bad and Bak in testicular tissue ($p<0.001$ in all cases), while the expression of anti-apoptotic gene Bcl2 was notably decreased ($p<0.001$). Supplementation with VC&VE restored the expressions of all these genes to near normal ($p<0.01$ in case of Bax, Bcl2 and Bad; $p<0.001$ in case of Bak). No noticeable difference was present between group I and IV (Figure 29).

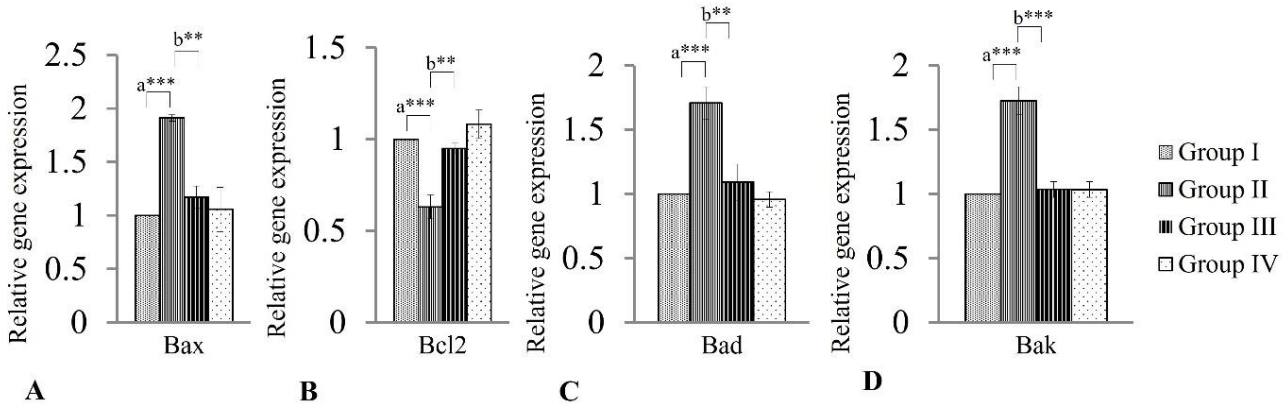

Figure 29: Bar diagram illustrating the analysis of relative gene expression of Bax (A), Bcl2 (B), Bad (C) and Bak (D) from testes of all four groups. Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; **p<0.01, ***p<0.001.

5.4.2 Effect of VC&VE on testicular protein level expressions of Bax and Bcl2 in fluoride treated rats

Analysis of immunoblotting clearly illustrated that fluoride considerably increased the expression of Bax in testicular tissue ($p<0.05$) while notably reduced the expression of Bcl2 ($p<0.05$). Interestingly, supplementing with VC&VE significantly decreased the expression of Bax ($p<0.05$). Improvement in Bcl2 expression was observed in supplemented group but not at the significant level. There was no noticeable distinction between groups I and IV in both the cases. (Figure 30).

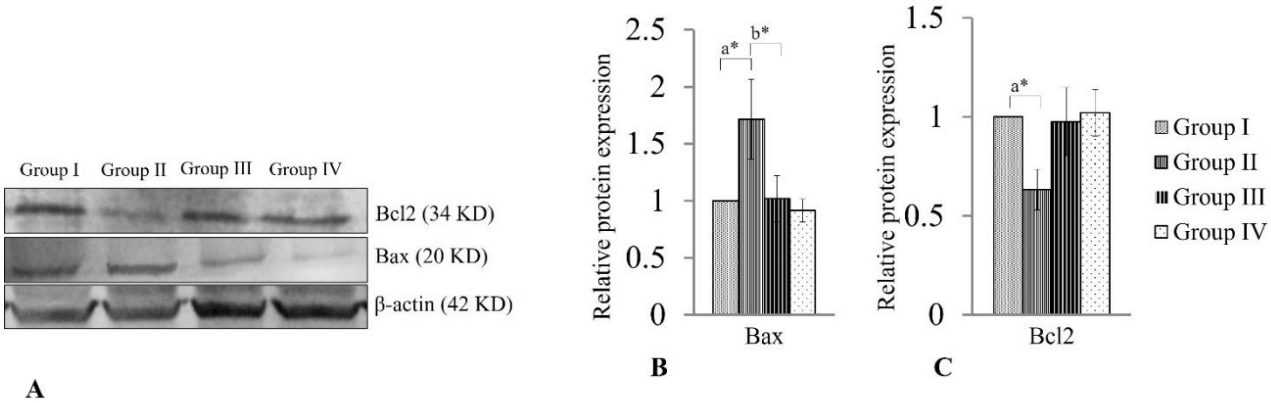

Figure 30: Testicular protein level expressions of Bax and Bcl2 were analyzed by Western Blotting and the consecutive densitometry analysis were performed. β-actin was used as loading control and densitometric analysis was done with reference to corresponding band of β-actin expression. (A) indicates band intensities of respective groups; (B) and (C) indicate relative protein expression of Bax and Bcl2 respectively. Values are presented as mean ± SEM,

n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; *p<0.05.

5.4.3 Effect of VC&VE on testicular protein level expressions of cleaved caspase 3 and cleaved caspase 9 in fluoride treated adult rats

In the testicular tissue of fluoride-treated rats, the relative protein expressions of cleaved caspase 3 and cleaved caspase 9 were noticeably increased (p<0.05 and p<0.001 respectively). The levels of these protein expressions were considerably reduced and achieved the normal value by the application of VC&VE (p<0.01 in both cases). There was no difference of relative protein expression between groups I and IV in both cases (Figure 31).

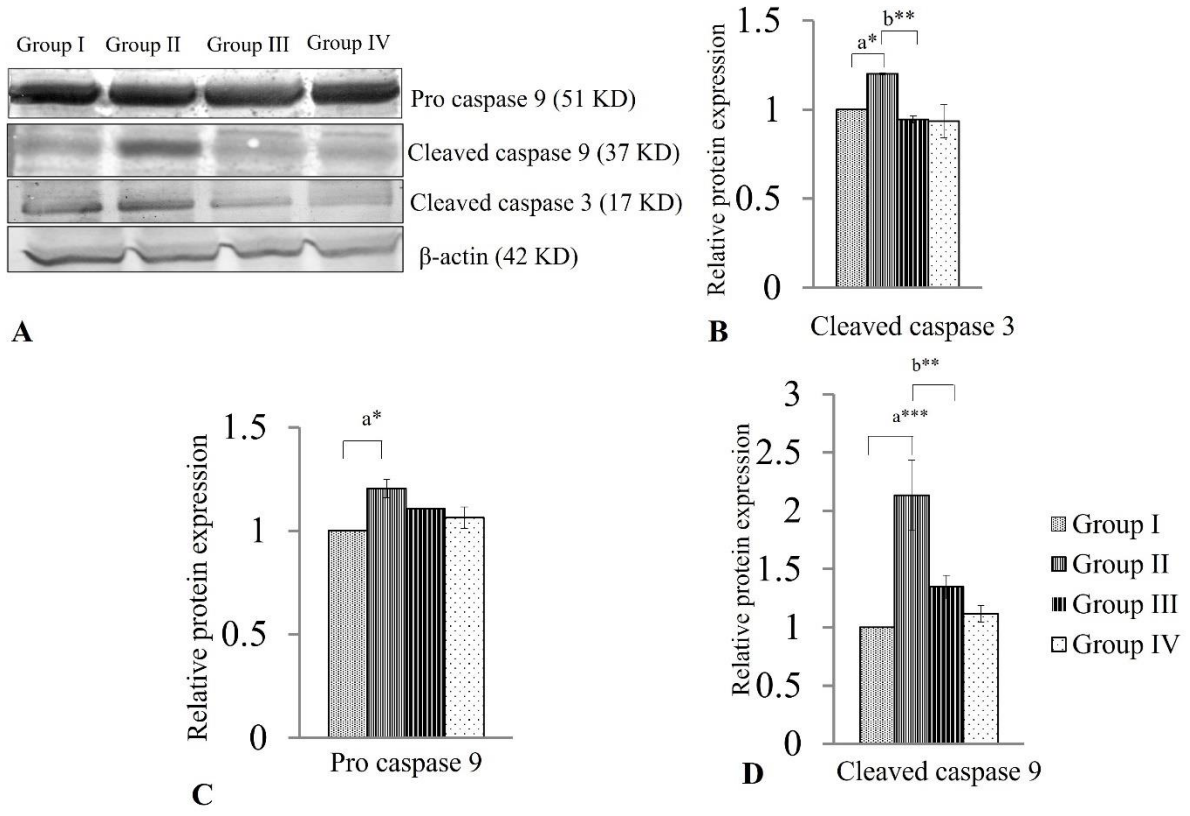

Figure 31: Testicular protein level expressions of cleaved caspase 3 and cleaved caspase 9 were analyzed by Western Blotting and the consecutive densitometry analysis were performed. β-actin was used as loading control and densitometric analysis was done with reference to corresponding band of β-actin expression. (A) indicates band intensities of respective groups; (B), (C) and (D) indicate relative protein expression of cleaved caspase 3, pro caspase 9 and cleaved caspase 9 respectively. Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; *p<0.05; **p<0.01; ***p<0.001.

5.4.4 Role of VC&VE on epididymal (cauda) mRNA expressions of Bax and Bcl2 genes in fluoride treated rats

Relative expressions of pro-apoptotic gene Bax was notably increased ($p<0.001$) in epididymal tissues after fluoride treatment but the expression of anti-apoptotic gene Bcl2 was significantly decreased ($p<0.01$). Supplementation markedly restored the expressions of these gene ($p<0.01$ in both cases). No difference was observed between group I and IV (Figure 32).

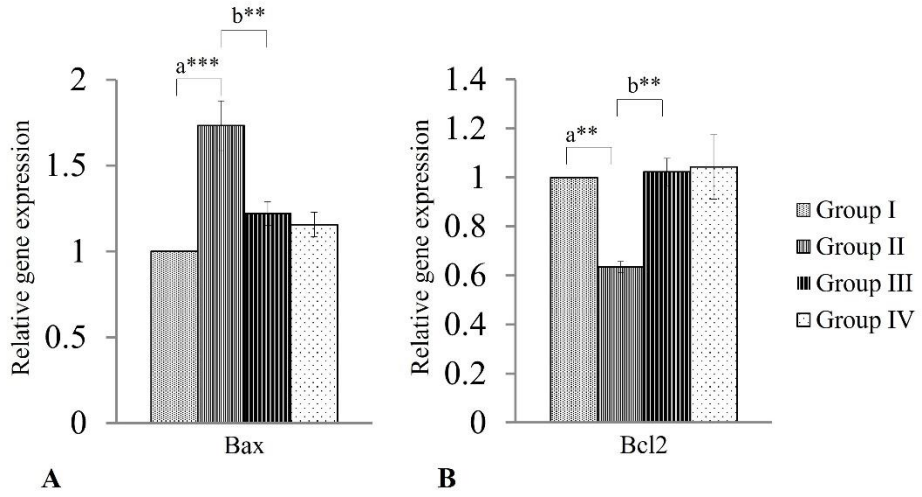

Figure 32: Bar diagram illustrating the analysis of relative gene expressions of Bax (A) and Bcl2 (B) from epididymis of all groups. Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; **p<0.01, ***p<0.001.

5.4.5 Role of VC&VE on epididymal (cauda) protein level expressions of Bax and Bcl2 in fluoride treated rats

Relative protein expression of Bax in epididymal tissue (cauda) was increased significantly ($p<0.001$) in fluoride treated rats, while such expression of Bcl2 was notably decreased ($p<0.001$). Supplementation with VC&VE meaningfully restored the expression of these proteins ($p<0.01$ in both cases) (Figure 33). No noticeable difference was present between group I and IV.

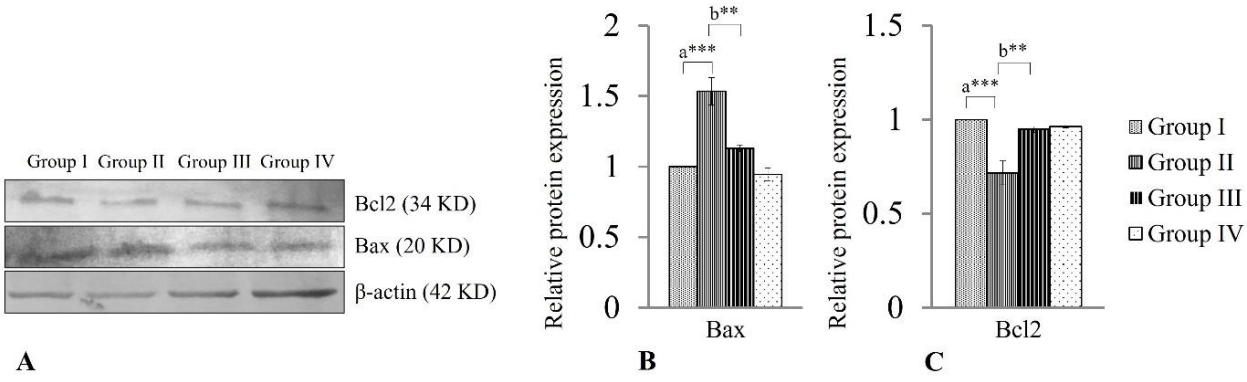

Figure 33: Epididymal protein level expressions of Bax and cleaved Bcl2 were analyzed by Western Blotting and the consecutive densitometry analysis were performed. β-actin was used as loading control and densitometric analysis was done with reference to corresponding band of β-actin expression. (A) indicates band intensities of respective groups; (B) and (C) indicate relative protein expression of Bax and Bcl2 respectively. Values are presented as mean ± SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; **p<0.01, ***p<0.001.

5.4.6 Role of VC&VE on epididymal (cauda) protein level expression of cleaved caspase 3 and cleaved caspase 9 in fluoride treated rats

Treatment with fluoride markedly enhanced the relative protein expressions of cleaved caspase 3 and cleaved caspase 9 in cauda epididymis ($p<0.001$ in both cases). Supplementation with VC&VE noticeably decreased such expressions ($p<0.001$ in both cases). No significant difference was present between group I and IV in both cases (Figure 34).

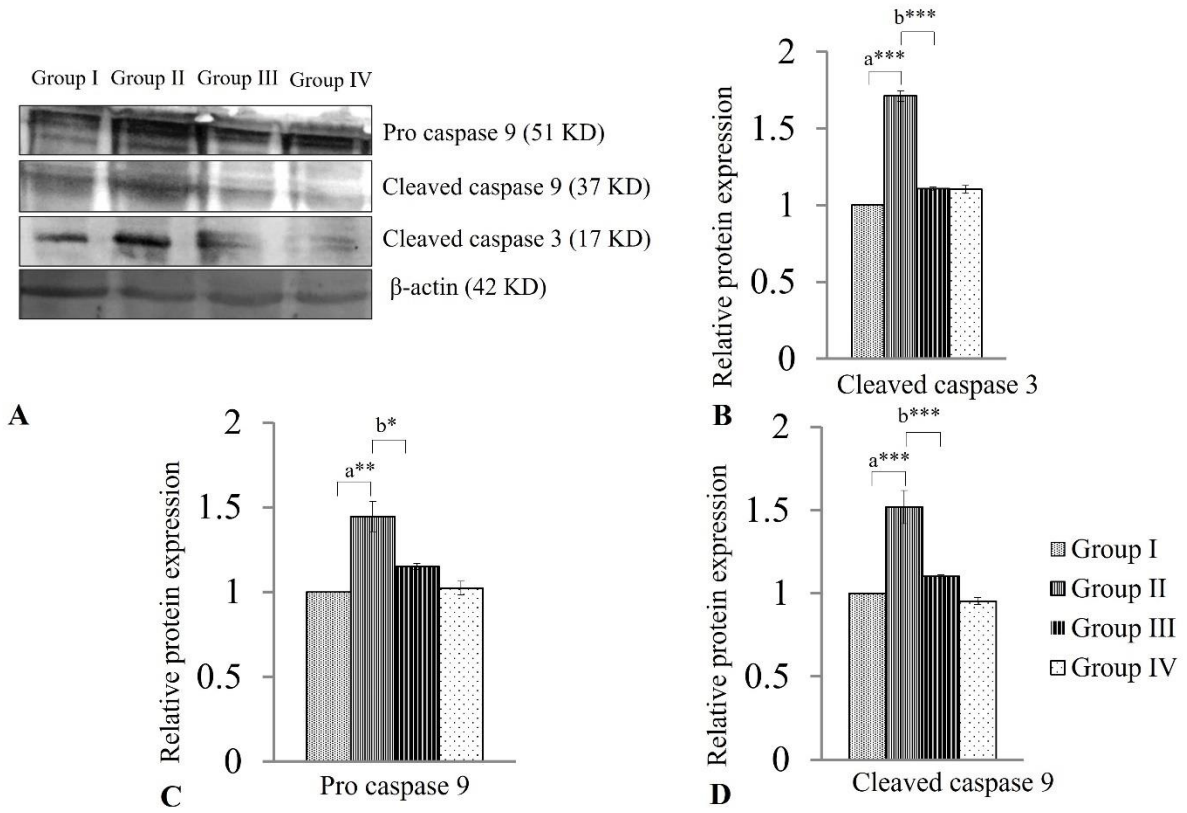

Figure 34: Epididymal (cauda) protein level expressions of cleaved caspase 3 and cleaved caspase 9 were analyzed by Western Blotting and the consecutive densitometry analysis were performed. β-actin was used as loading control and densitometric analysis was done with reference to corresponding band of β-actin expression. (A) indicates band intensities of respective groups; (B) and (C) indicate relative protein expressions of cleaved caspase 3 and cleaved caspase 9 respectively. Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; ***p<0.001.

5.4.7 Effect of VC&VE on spermatozoal apoptosis in fluoride treated rats

Application of NaF enhanced the percentage of early apoptotic spermatozoa ($p<0.01$) decreased the percentage of necrotic spermatozoa ($p<0.001$) in case of caput epididymis but no noticeable change in the percentage of late apoptotic spermatozoa was noted. Marked reduction of the early apoptotic ($p<0.01$) spermatozoal percentage was observed after supplementation with VC&VE though a significant difference in the percentage of necrotic spermatozoa was existed between group I and group III. No noticeable change in these parameters was seen between group I and group IV (Figure 35, Tabel 17).

Significant increased percentage of early apoptotic ($p<0.01$), late apoptotic ($p<0.001$) and necrotic ($p<0.05$) spermatozoa isolated from cauda epididymis was observed in fluoride treated rats. However, supplementation exhibited notable recovery in all cases ($p<0.05$, $p<0.001$ and

p<0.01 respectively) but significant difference still existed between control and supplemented group in case of early apoptotic spermatozoa. No significant alteration was observed between group I and IV (Figure 36, Table 18).

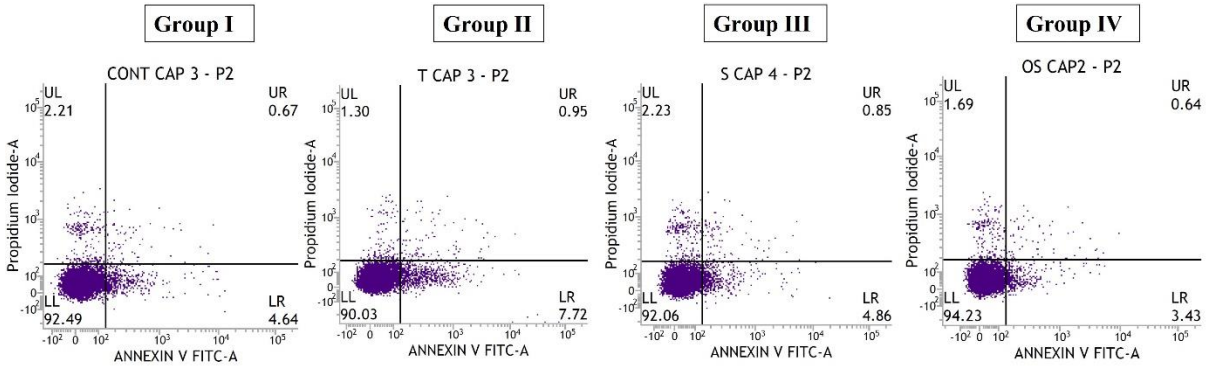

Figure 35: The flow cytometric profile of caput spermatozoa in respective groups that are labeled with Annexin V and PI depicting the percentage of live, early apoptotic, late apoptotic and necrotic spermatozoa

Table 17: Effect of VC&VE supplementation on spermatozoal (caput) apoptosis caused by fluoride toxicity in adult male rats

Animals	Early apoptotic spermatozoa %	Late apoptotic spermatozoa %	Necrotic spermatozoa %
Group I	4.19±0.499	0.67±0.156	2.20±0.043
Group II	7.30±0.432[a**]	0.79±0.090	1.08±0.125[a***]
Group III	4.31±0.544[b**]	0.65±0.123	2.17±0.197[b***]
Group IV	3.33±0.530	0.61±0.136	1.89±0.070

Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; **p<0.01, ***p<0.001.

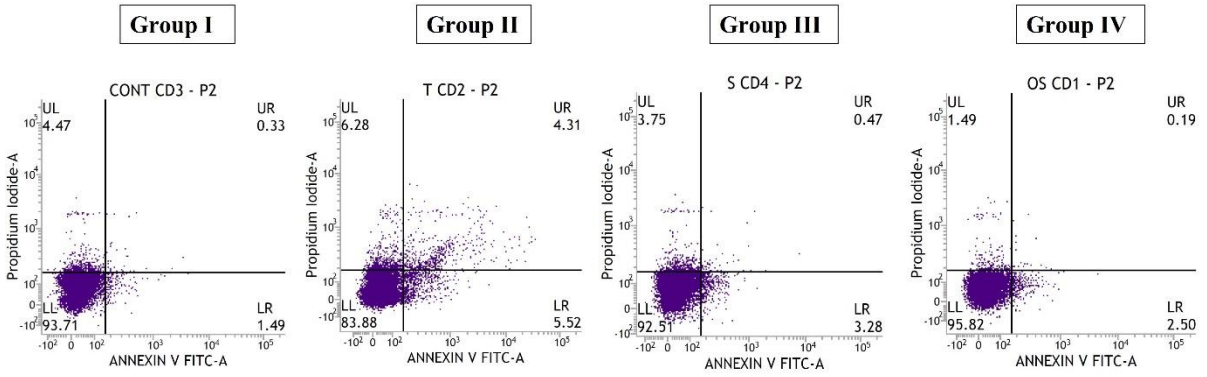

Figure 36: The flow cytometric profile of cauda spermatozoa in respective groups that are labeled with Annexin V and PI depicting the percentage of live, early apoptotic, late apoptotic and necrotic spermatozoa.

Table 18: Effect of VC&VE supplementation on spermatozoal (cauda) apoptosis caused by fluoride toxicity in adult male rats

Animals	Early apoptotic spermatozoa %	Late apoptotic spermatozoa %	Necrotic spermatozoa %

Group I	1.44±0.174	0.30±0.106	4.21±0.951
Group II	5.40±0.322^{a**}	4.28±0.054 ^{a***}	7.15±0.598^{a*}
Group III	3.75±0.987^{a*b*}	0.69±0.267^{b***}	2.87±0.464^{b**}
Group IV	2.64±0.057	0.31±0.047	2.49±0.442

Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; *p<0.05, **p<0.01, ***p<0.001.

5.4.8 Effect of VC&VE on testicular mRNA expression of TNF-α gene in fluoride treated adult rats

Relative gene expression on testicular TNF-α was increased significantly (p<0.001) in fluoride treated rats, while supplementation notably (p<0.001) restored this level. No noticeable change in this gene expression was observed between group I and IV (Figure 37).

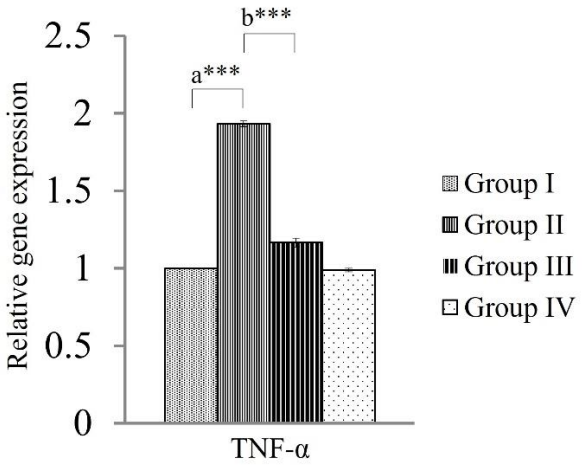

Figure 37: Bar diagram illustrating the analysis of relative gene expressions of TNF-α in testicular tissue from all groups. Values are presented as mean±SEM, n=8 and comparisons are made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; ***p<0.001.

5.4.9 Effect of VC&VE on testicular protein level expressions of NF-κB and TNF-α in fluoride treated adult rats

Immunoblotting analysis firmly revealed that fluoride significantly enhanced the expression of NF-κB (p<0.001) and TNF-α (p<0.01) in testicular tissues. Supplementation with VC&VE notably restored the levels of these protein (p<0.01 in both cases). No significant difference was observed between group I and IV (Figure 38).

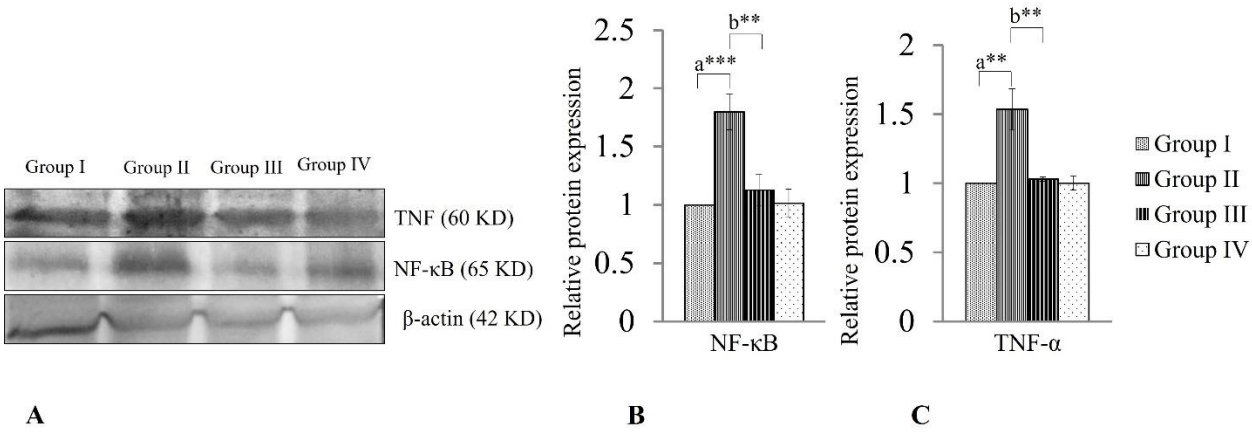

Figure 38: Testicular protein level expressions of NF-κB and TNF-α were analyzed by Western Blotting and the consecutive densitometry analysis were performed. β-actin was used as loading control and densitometric analysis was done with reference to corresponding band of β-actin expression. (A) indicates band intensities of respective groups; (B) and (C) indicate relative protein expression of cleaved NF-κB and TNF-α respectively. Values are presented as mean±SEM, n=8 and comparison is made by *Tukey's test*. a = group I vs group II, III & IV; b = group II vs group III; **p<0.01; ***p<0.001.

6. Discussion:

The goal of the current study was to investigate the adverse impacts of NaF on the male reproductive system, taking into consideration several aspects such as structure-function aberrations of the testis, epididymis, and spermatozoa, as well as their altered genomic and proteomic status. Additionally, this study was further designed to provide insight on the extent of the protective effects of VC&VE against fluoride-mediated reproductive disorders.

The study (results obtained from objective 1) clearly showed that the extent of toxicity on the male reproductive system of adult Wistar rats increased with increasing dosages of NaF. Exposure to NaF in graded doses (10, 15, and 20 mg/kg body weight/day) for 30 consecutive days evidently decreased the body weight in group III (NaF at a dose of 15 mg/kg body weight/day) and IV (NaF at a dose of 20 mg/kg/day) rats. Marked weight loss of the testis was also seen in these groups, but there were no notable weight changes in the epididymis, prostate and seminal vesicle. Androgen is crucial for reproductive organ structure and function, as well as for maintaining body weight, and its accessibility is closely associated with organ weight[56]. Exposure to fluoride limits the synthesis of testicular steroids, which subsequently lowers the availability of androgen[57]. Consequently, decreased body weight and testicular weight may be caused by reduced steroidogenic activity.

Healthy structural integrity of the testis and epididymis is obligatory for spermatogenesis, spermatozoal maturation, and several physiological functions at an optimal level. The normal histological structure of the testis is characterised by well-organized round or oval seminiferous tubules, intact basement membrane of seminiferous tubules, successive developmental stages of spermatogonial cells within seminiferous tubules and intact Leydig cells. Our study showed that group III and IV (15 and 20 mg/kg/day respectively) rats significantly displayed testicular degenerative changes, such as disorganised seminiferous tubules, distorted basement membranes, and a lack of subsequent stages of spermatogonial cells, but group II (10 mg/kg/day) rats did not show these changes to the same degree.

The presence of rounded lobules covered by epithelium with spermatozoa inside the lumen and stereocilia on the inner surface of the epithelium are considered structural characteristics of the epididymis (both caput and cauda). NaF caused structural damages to the epididymis, resulting in a reduction in spermatozoal concentration and also a thinning of the epithelial layer which were more pronounced in the cauda than that of the caput. These structural abnormalities were mostly noticeable in the cauda of rats in groups III and IV, but only in group IV in the case of the caput. It is clear from the literature that fluoride may damage the blood-testes barrier, have harmful effects on the development and differentiation of spermatogonial cells as well as on the stoppage of spermatogenesis[58]. It is also known that fluoride inhibits testosterone synthesis, which is essential for the development and operation of the male reproductive system[57]. These might be reasonable explanations for altered testicular and epididymal histoarchitecture.

The spermatozoal potential, *i. e.* count, motility, viability, and plasma membrane integrity, were affected by the gradual increment of NaF doses. With the exception of spermatozoal viability, which was considerably decreased starting from group III, all of these characteristics were significantly and dose-dependently altered. Literature states that sufficient zinc concentration within testis and optimum activity of angiotensin-converting enzyme are responsible for purposeful life span of spermatozoa and also for their development[59], and fluoride is responsible for reduction of serum testosterone level and testicular zinc concentration, which in turn causes the decrease of the activity of the angiotensin-converting enzyme and diminution of spermatogenesis[60]. This might be a possible explanation for the drop in sperm count following the fluoride intoxication. Spermatozoal motility is necessary for effective fertilisation. Two essential components are for sperm motility are adequate fructose content in seminal vesicle and androgen carrier protein. Spermatozoal motility is impaired by fluoride-mediated reductions in androgen carrier protein concentration and seminal vesicle

fructose content[61,62]. It is widely recognised that normal structural integrity of epididymis is crucial for the production of mature and viable spermatozoa, and any change in this aspect may be considered fatal for spermatozoal viability. This study showed that fluoride disrupts the structural integrity of epididymis, which in turn reduces the number of viable spermatozoa. The functional integrity of the spermatozoal plasma membrane, a reliable indicator of successful fertility, is generally assessed using HOS test. As a result of the inflow of hypo-osmotic fluid across the sperm plasma membrane during this test, the sperm tail coils. The more sperm with an intact plasma membrane there are, the higher the coiled tail percentage. Fluoride promotes spermatozoal ROS formation resulting redox imbalance. Due to the presence of a significant quantity of polyunsaturated fatty acids in their plasma membrane, spermatozoa are susceptible to lipid peroxidation, which results in non-functional entity. Therefore, the elevated ROS production brought on by fluoride poisoning results in compromised spermatozoal plasma membrane functional integrity.

Redox equilibrium is recognised to be essential for the healthy physiological functioning of all systems. For the purpose of restoring this equilibrium, various internal antioxidant molecules fight against ROS as they are generated in various systems as a result of cellular metabolism at various levels. Our findings suggest that a rise of NaF, disturbed the normal redox balance of testes due to an insufficient antioxidant defence mechanism, resulting in a testicular oxidative damage. Superoxide radicals are scavenged by SOD, one of the enzymatic markers, into hydrogen peroxide, which is then broken down by catalase, another marker, into water and oxygen. Together with GSH, an internal antioxidant, another enzyme, GPx converts hydrogen peroxide to water. Finally, GST is crucial for the conjugation of electrophilic molecules (produced during the metabolism of xenobiotics) with GSH for excretion from the body. Both protein oxidation, which results in the generation of PCs, and membrane lipid (polyunsaturated fatty acids) peroxidation which results in the production of MDA are ROS-mediated cellular impairments. Increased levels of MDA and PC are regarded as nonenzymatic indicators of oxidative stress caused by ROS. Literature states that exposure to fluoride increases the formation of superoxide, nitric oxide, and lipid peroxide radicals while decreasing the activity of antioxidant system[63]. In our findings, we have seen the alteration of enzymatic and non-enzymatic markers of oxidative stress in testis, indicating an increase in redox imbalance in a dose-dependent manner upon NaF administration.

This study makes it clear that both structural and functional changes in the testis are brought on by fluoride-mediated oxidative stress. ACP and ALP are considered as vital biomarkers of

primordial germ cells. Early spermatogenic development is characterised by the normal activity of ALP, which aids in cell division and glucose delivery to spermatogenic cells[64]. Additionally, it has been shown that ALP plays a vital role in the production of testosterone, which is essential for the early stages of spermatogenesis and also for the development of spermatids[65]. This study demonstrated that, beginning from group III, ACP and ALP activities were severely hindered, adding to the evidence for altered androgenesis and spermatogenesis in the presence of NaF. In oxygen-limited conditions, LDH is an enzyme that drives glycolysis by converting pyruvate to lactate via a NADH-dependent route. Glycolysis is the main energy metabolic activity in the testis, and LDH is regarded as a crucial enzyme for energy metabolism of spermatogonial cells[66]. This study showed that LDH activity was gradually decreased when NaF dosages were increased gradually in the treated groups, indicating a decreased source of energy that caused the disintegration of spermatozoa.

Under any xenobiotic stress, ROS-induced DNA damage, as expressed by increased DNA fragmentation, predominates[66]. According to our study, fluoride-induced ROS-mediated oxidative stress, which was resulted in a dose-dependent manner, is the main cause of testicular DNA fragmentation.

DNA and nucleoproteins make up the highly ordered and compact structure of spermatozoal chromatin. In the later stages of spermatogenesis, the histone protein of sperm nucleus is replaced by the basic cysteine-rich nucleoprotein protamine. Present study using AO staining divulges fluoride mediated altered spermatozoal DNA integrity as evidenced by reduced percentage of green fluorescent spermatozoa. Intact native DNA is indicated by green fluorescent spermatozoa, whereas denatured DNA is indicated by red fluorescent spermatozoa. Here, the higher percentage of red fluorescent spermatozoa unequivocally demonstrates the frequency of spermatozoal DNA strand breaking in the groups that received NaF treatment. Fluoride has adverse impacts through increasing oxidative stress, and spermatozoa are vulnerable to lipid peroxidation because of the high concentration of polyunsaturated fatty acids in their plasma membrane and the dearth of ROS scavengers[67]. Therefore, it may be postulated that changed spermatozoal DNA state may be caused by increased ROS formation.

These experimental arrays clearly showed that, among the three dosages, the 15 mg/kg dose (group III) exhibited the most negative effects as a minimal dose. Therefore, for fulfilment of the rest objectives (objective 2, 3 and 4), which focused on ameliorative potential of VC&VE against reprotoxic effects of fluoride, this dose has been chosen.

Despite receiving fluoride treatment, the addition of VC&VE significantly restored testicular weight. Maintaining the weight of the reproductive organs requires the presence of androgen. Literature states that vitamin C is essential for the maintenance of testicular integrity and steroidogenic activities[68]. Rats with vitamin E deficiency have degenerative abnormalities in their testicles[69]. Therefore, it can be assumed that supplementation of VC&VE has the potency to recover testicular degenerative changes.

Present study showed that fluoride exerted deleterious effects in the structural integrity of testis in terms of disorganized seminiferous tubules, absence of developmental stages of spermatogonial cells, decreased seminiferous epithelial height, increased luminal diameter of seminiferous tubule and decreased Leydig cell nuclear area. Ultra-structural analysis further conferred that fluoride caused partial degeneration of seminiferous tubules, disarray in basement membrane and disorientation of spermatogenic cells. However, supplementation exhibited noteworthy recovery from such disadvantageous outcomes. Normal serum testosterone level is necessary for testicular tissue growth and function; however, fluoride lowers this level, which has an impact on testicular growth. This might be the most likely causes underlying the degradation of testicular structure. According to the literature, VC is more effective than VE at maintaining blood testosterone levels in lead-exposed rats, but the combined effects of these two are far more effective than single treatments[70]. In this study, it was also seen that the altered steroidogenic stratum caused by fluoride had been recovered with the addition of VC&VE. Therefore, it can be concluded that combined application of VC&VE restored the steroidogenic state which in turn maintains normal testicular growth and structural integrity.

Present study clearly divulged that fluoride caused reduced testicular antioxidant defence mechanism as evidenced by decreased activities of SOD, catalase, GST, GPx along with declined GSH content. Provoked oxidative stress was further reflected by augmented MDA and PC contents. However, supplementation with VC&VE restored the activities of all antioxidants considerably. MDA and PC contents also reduced considerably upon supplementation. Through the reduction of one electron of the lipid hydroperoxyl radicals via the vitamin E redox cycle, vitamin C acts as an antioxidant and scavenge reactive oxygen species (ROS) to prevent lipid peroxidation[24]. Vitamin E is an effective peroxyl radical scavenger and a chain-breaking antioxidant that prevents the formation of free radicals in membranes and plasma lipoproteins. Hydroxyl group of the tocopherol reacts with peroxyl radical forming tocopheryl radical (Vit E-O) along with lipid hydroperoxides and finally

tocopheryl radical reacts with vitamin C and return to its reduced state. So, it can be assumed that vitamin C and E synergistically scavenges free radicals and aggravates internal antioxidants. When a cell experiences stress, Nrf2 detaches from Keap1, a regulator of Nrf2, moves to the nucleus and binds to various components involved in antioxidant defence[71]. The present study showed that fluoride reduced testicular Nrf2 level, which further supports the evidence of decreased antioxidant status in the treated group. VC&VE supplementation reinstated the level of Nrf2, which in turn maintained the redox status of the testis.

Fluoride-induced oxidative stress also altered the functional strata of the testicles, as seen by decreased ACP, ALP, and LDH activities. The functional activity of these marker enzymes was significantly recovered by VC&VE supplementation. Testicular ACP and ALP are two significant biomarkers that support the development of primordial germ cells; additionally, ALP encourages glucose transportation and the division of spermatogenic cells[64]. LDH is an enzyme that aids in glycolysis by converting pyruvate to lactate via a NADH-dependent route and it is also responsible for the energy metabolism in spermatogonial cells. Following fluoride intoxication, the quenched activities of these functional marker enzymes clearly indicate the alteration of spermatogenesis. However, by virtue of the antioxidant properties of VC&VE these functions were restored.

This study revealed fluoride mediated altered testicular steroidogenesis and diminished gonadotrophin level whereas supplementation exhibited noteworthy recovery from quenched steroidogenesis and reduced gonadotrophins levels. Key regulatory enzymes of testicular steroidogenic pathway are 3ß-HSD and 17β-HSD. Earlier study reported that fluoride causes reduced genomic expressions of these markers[72]. Literature further revealed that genomic expression of FSH and LH receptors is suppressed by fluoride[73]. One previous study also demonstrated that vitamin C and E restored serum FSH and LH contents along with mRNA expression of 3β -HSD and 17β-HSD in polychlorinated biphenyl exposed adult rats[74].

Every human cell experiences DNA damage as a result of oxidative stress caused by ROS[75]. The fragmented genomic DNA in this study clearly demonstrated fluoride-mediated testicular DNA damage, which may be brought about by enhanced ROS-associated oxidative stress. Comet assay had also strengthened the occurrence of testicular DNA damage as evidenced by presence of increased comet tail length, tail moment and olive tail moment. The addition of VC&VE led to a notable recovery of such adverse effects. According to similar research report, vitamin C protects against DNA damage caused by oxidative stress by regulating DNA repair

enzymes[76]. Vitamin E can mitigate the effects of DNA damage by lowering the amount of the DNA damage marker 8-hydroxydeoxyguanosine[77]. Thus, it can be concluded that VC and VE can protect NaF mediated testicular DNA damage as evidenced by the absence of any prominent trail of fragmented DNA along with decreased comet tail length, tail moment and olive tail moment in supplement group.

The blood testis barrier (BTB) is a physical barrier that prevents the passage of a number of exogenous and endogenous toxicants, and its malfunction results in toxicant-mediated diseases of the male reproductive system. According to published research, fluoride increases the expression of BTB-associated proteins such actin relative protein 3 and interleukin-1 alpha, which can harm the ultra-structure of BTB[78]. Enhanced expression of IL-1a in turn decreases the expression of filamentous actin which maintain structure function integrity of BTB. Therefore, it can be said that the overexposed fluoride can cross BTB which was evidenced by increased accumulation of fluoride in testis. Further investigations are needed to conclude the role of VC&VE on BTB and its associated proteins.

Although spermatogenesis is exclusively dependent on close liaison in-between Sertoli and Leydig cells of testis but the spermatozoa formed by the testis are immature and infertile. For the spermatozoa to fully develop and become fertile, they must be carried via the epididymis which is a unique and fascinating accessory sex organ of the male reproductive system made up of super coiled and convoluted tubule restraining a nutritious luminal fluid required by the spermatozoa for the attainment of fertility. Epididymis is divided into three separate regions based on structural orientation, including caput, corpus, and cauda. In this study, I looked at a variety of criteria to evaluate the protective potential of VC&VE against fluoride-induced altered structure-function integrity of the caput and caudal areas of the epididymis as well as isolated caput and caudal spermatozoa.

Histology and SEM showed that fluoride poisoning causes degenerative alterations in both sections of the epididymis, however the cauda region is more vulnerable than the caput. Supplements significantly aided in recovering from such structural anomalies. The structural integrity of reproductive organs depends on an adequate androgen supply, which is restricted by fluoride intoxication whereas vitamin C and E can considerably increase androgen levels. In this manner, supplementing restored the structural integrity of the epididymis.

Apart from structural anomalies, fluoride also caused epididymal redox imbalance having a greater impact on the cauda region than the caput. Only the catalase activity was dramatically

reduced in caput region, although the cauda showed noticeably changed expression of all oxidative stress indicators. The presence of oxidative stress in fluoride-treated animals was further confirmed by immunoblotting analysis of Nrf2, however supplementation restored the lower expression of this marker in treated rats. However, supplementation notably restored the activities of all the markers. Being a biologically and chemically active component, fluoride may quickly permeate cellular membranes and exert its harmful effects by inducing oxidative stress. Excess ROS generation and decreased antioxidant defence are all linked to fluoride toxicity[13]. The antioxidant characteristics of the selected supplement slowed down ROS production and reduced oxidative stress.

Exogenous sources, such as UV light and ionising radiation, as well as endogenous triggers, such as mistakes in DNA replication and oxidative stress, can both result in DNA damage. Increased amounts of ROS can cause DNA oxidation, especially in oxidised DNA bases, which is another significant cause of DNA damage[79]. This study clearly showed that excessive oxidative stress in the cauda area led to DNA fragmentation, as seen by a ladder of fragmented DNA in fluoride-treated rats, which was not found in the caput region due to a lack of provoked oxidative damage. The application of VC&VE led to a notable recovery of such harmful effects due to their protective ability against oxidative stress mediated DNA damage.

Fluoride caused structural abnormalities of spermatozoa as evidenced by eosin/nigrosine staining as well as by SEM. Fluoride exposure resulted in a notable rise in the proportion of both primary and secondary malformations, whereas supplementation decreased the percentage of aberrant spermatozoa. Several genes, including Sly, Ssty2 (expressed during spermatogenesis and situated on the male-specific region of the Y chromosome), and HSF2, are necessary for the spermatozoal head to maintain its structural integrity. Fluoride inhibits the expression of these genes at the genomic and proteomic levels[80], which might result in defects in the sperm head. The 9+2 arrangement of microtubules is necessary for the structural integrity of spermatozoal flagella. Eleven important regulatory genes are connected with the preservation of spermatozoal flagella among which fluoride changes the expression of five genes, including AKAP3, AKAP4, CFAP43, CFAP44, and HYDIN[81], which could be a plausible cause of fluoride-mediated defects in spermatozoa tail. Based on recent study, vitamins C and E improve sperm morphology, count, and progressive motility in the human population[82]. Experimental study on rat model also divulged that vitamin C and E can improve sperm concentration, motility and morphology in smoke induced rats[83]. Therefore, it can be

concluded that VC&VE has the potential to restore the sperm morphology in fluoride treated rats.

Application of VC&VE alleviated fluoride mediated altered sperm potential in both caput and cauda regions. VC&VE can improve fluoride-mediated reduced sperm concentration by raising testicular zinc level since an earlier investigation revealed that the combined action of VC and VE preserves testicular zinc level[70]. Spermatozoal motility is sturdily correlated with both androgen carrier protein and fructose content of seminal vesicle and previous study demonstrated that combined action of VC and VE upsurges seminal vesicle fructose content[84] ,which in turn restores sperm motility in fluoride treated rats. The chosen supplement also maintained the structural integrity of epididymis which in turn reinstated the number of viable spermatozoa. The combined use of VC&VE enhances spermatozoal plasma membrane functional integrity by lowering excessive ROS generation.

Prolonged F exposure results in oxidative damage. Due to the abundance of polyunsaturated fatty acids in their plasma membranes, spermatozoa are particularly susceptible to lipid peroxidation. Low levels of ROS-scavenging enzymes, which are present in the cytoplasm of spermatozoa, may not be adequate to counteract the increased ROS generation brought on by oxidative stress[67]. This study also depicted that fluoride causes spermatozoal ROS generation in both the caput and cauda regions of the epididymis. Despite receiving fluoride treatment, the ROS scavenging capacity of both VC and VE decreased the oxidative damage in spermatozoa.

DNA and nucleoprotein make up the highly ordered and compact structure of spermatozoal chromatin. In the latter stages of spermatogenesis, the sperm nucleus histone protein is replaced by the protamine, a basic nucleoprotein that is rich in cysteine. Our study clearly pointed out that fluoride toxicity is responsible for a decreased proportion of protamine rich mature spermatozoa, as evidenced by an increased percentage of aniline blue positive sperm. The study using acridine orange further confirmed fluoride mediated alterations of spermatozoal DNA integrity, as evidenced by a decreased percentage of green fluorescent spermatozoa. The higher proportion of red fluorescent spermatozoa in this instance strongly supports the results of aniline blue staining by demonstrating the frequency of spermatozoal DNA strand breaks in the NaF-treated group. The primary cause of the DNA damage is an excess of ROS produced by fluoride in spermatozoa. However, the supplementation of VC&VE effectively reversed the spermatozoal abnormalities, as evidenced by a decrease in the proportion of aniline blue-positive spermatozoa and an increase in the proportion of green fluorescent spermatozoa. The

antioxidant capacity of VC&VE is likely how they combat the detrimental effects of oxidative stress, which in turn reduces fluoride-induced spermatozoal DNA scratch.

The constant need for DNA repair to maintain genomic integrity is evidence that DNA damage is a widespread occurrence in life. The apoptosis machinery is also widespread and can be found latently in the majority of the cells of multicellular animals. A substantial amount of evidence supports the relationship between DNA damage and apoptosis[85]. In this study, I also studied the expression of few genes and proteins involved in the apoptotic pathway in fluoride induced state, along with the protective ability of chosen supplements in testis and epididymis. Many proteins are known to be involved in the apoptotic process, among which Bcl2 is involved in the antiapoptotic pathway and Bax, Bad, Bak, Caspase 3, and Caspase 9 are involved in the pro-apoptotic pathway. This study clearly revealed that fluoride increased the occurrence of apoptosis in the testis and cauda epididymis, as demonstrated by increased genomic and proteomic expression of pro-apoptotic genes while decreased expression of anti-apoptotic genes. Increased DNA damage caused by fluoride may be a plausible reason of apoptosis. Supplementation restored the expression of all these marker genes in testis and epididymis. One previous report suggested that vitamin C and E shows reversible effects against oxidative stress induced apoptosis in melamine treated PC12 cells[86]. Due to its antioxidative properties, vitamin C and E significantly lower the amount of reactive oxygen species produced in mitochondria, which are necessary for the execution of fluoride-induced apoptosis.

This study also revealed that fluoride enhanced the spermatozoal apoptosis in both caput and cauda regions of epididymis as evidenced by increased percentage of early apoptotic, late apoptotic and necrotic spermatozoa. VC&VE was also effective in preventing such disparaging effect of fluoride. Human ejaculated spermatozoa exhibit several apoptotic indicators like caspase activation, externalisation of phosphatidyl serine, change in mitochondrial membrane potential, and DNA fragmentation. Additionally, ROS are also crucial in the many forms of male infertility. One of the well-known processes of testicular quality control is apoptosis. However, an increase in apoptosis can be harmful for sperm production. Recent research has demonstrated that ROS and the resulting oxidative stress cause apoptosis[87]. This study also showed that fluoride increased the production of ROS in spermatozoa, which may be a possible explanation for apoptosis, while the antioxidant properties of chosen vitamins reduced the percentage of apoptotic spermatozoa by quenching ROS generation.

Regulation of testicular steroidogenesis and spermatogenesis as well as the maturation of semen are under the control of some inflammatory mediators such as NF-κB, TNF-α, interleukin-1β (IL-1β), iNOS and cyclooxygenase-2 (COX-2)[19]. This study showed that genomic expression of TNF-α and proteomic expressions of TNF-α and NF-κB increased due to fluoride exposure, which depict testicular inflammation that in turn hamper testicular steroidogenesis and spermatogenesis. However, supplementation with VC&VE restored the expressions of such markers. Literature states that vitamin C demonstrates its antioxidant and anti-inflammatory properties, beginning with the direct scavenging of free radicals and the suppression of pro-inflammatory pathways, moving on to the activation of intracellular antioxidant systems, and finally assisting the action of other exogenous antioxidants[88]. Literature also revealed that one of the main known pro-inflammatory signalling pathways affected by vitamin C is the NFκB/TNFα pathway[89]. Another report revealed that vitamin E supplementation significantly lowered the inflammatory markers such as C-reactive protein and TNF-α in patients with coronary artery disease[90]. Therefore, it can be concluded that VC&VE can efficiently attenuate fluoride mediated inflammatory response in testicular tissues.

7. Conclusion:

This study concluded that fluoride, being an environmental contaminant, impaired the structural organization and hindered the functional coherence of male reproductive organs. As studied the testis, epididymis and spermatozoa were seen to be affected. Beyond oxidative damages, steroidogenic, apoptotic and inflammatory pathways were hampered by overexposure to fluoride as evidenced by histological, biochemical, genomic and proteomic analyses. Despite fluoride treatment, supplementation with VC&VE alleviated all these discrepancies. Antioxidant, antiapoptotic as well as anti-inflammatory properties of these vitamins might be responsible for such amelioration.

8. Significance of this study:

The hazardous effects of fluoride can affect people from all around the globe. The negative effects of fluoride on the male reproductive system were highlighted in this study. Despite the fact that this study used a rat model, the findings are quite alarming. Therapeutic strategies of

vitamin C and E are also quite intriguing. Therefore, one way to counteract the negative effects of fluoride would be to take these readily accessible, inexpensive natural antioxidants.

9. Acknowledgement:

I am grateful to my supervisor for constant motivation and support to complete this work. I am thankful to my lab mates Sagnik Biswas, Rubia Mondal and Jeet Maity for helping me to carry out this study. I am grateful to FRPDF grant of Presidency University, DST-FIST and DBT Builders grants. Scanning electron microscopy and FACS were performed at Centre for Research in the Nanoscience and Nanotechnology, University of Calcutta. Assessment of testicular fluoride content by ion selective electrode was performed at the School of Environmental Studies, Jadavpur University and for this I am also grateful to the director of School of Environmental Studies, Jadavpur University Dr. Tarit Roy Chowdhury.

10. Abbreviation:

AB: Aniline blue

ACP: Acid phosphatase

ALP: Alkaline phosphatase

AO: Acridine orange

CAT: Catalase

COX-2: Cyclooxygenase 2

DCFDA: 2,7- dichlorodihydrofluorescein diacetate

DMSO: Dimethylsulfoxide

DNPH: 2,4-dinitrophenylhydrazine

ELISA: Enzyme linkedimmunosorbent assay

ER: Endoplasmic reticulum

FACS: Fluorescent activated cell sorter

FSH: Follicle stimulating hormone

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: Glutathione

GST: Glutathione S-transferase

H/E: Hematoxylin-eosin staining

HOST: Hypo-osmotic swelling

HSD: Hydroxysteroid dehydrogenase

IL: Interleukin

LDH: Lactate dehydrogenase

LPO: Lipid peroxidation

LSD: Least significant difference

MDA: Malondialdehyde

NaF: Sodium fluoride

NMS: Non-motile sperm

NPMS: Non-progressively motile sperm

NRF2: Nuclear factor erythroid 2–related factor 2

PC: Protein carbonyl

PMS: Progressively motile sperm

ROS: Reactive oxygen species

SOD: Superoxide dismutase

SEM: Scanning electron microscopy

TNF-α: Tumor necrosis factor alpha

11. Appendix:

Table 19: Buffer composition

Serial Number	Buffer Name	Composition	Used in
1	Hypo-osmotic solution	7.35 g sodium citrate and 13.51 g fructose in 1 L distilled water	Hypo-osmotic swelling (HOS) test
2	Tissue homogenization buffer	0.1 M phosphate buffer (pH 7.4)	MDA
3	TBA-TCA mixture	0.5 mL saline (0.9%) and 2 ml of 2-thiobarbituric acid-trichloroacetic acid (TBA– TCA) mixture (0.392 g TBA in 75 ml of 0.25 N HCl with 15 g TCA in a volume made up to 100 ml by 95% ethanol)	MDA
4	Tissue homogenization buffer	50 mM potassium phosphate buffer (pH 7.4)	Protein carbonyl content
5	Tissue homogenization buffer	0.1 M phosphate buffer (pH 7.2)	SOD
6	Tissue homogenization buffer	0.05 M Tris–HCl (pH 7.0)	Catalase
7	Tissue homogenization buffer	50 mM phosphate buffer (pH-7.4)	GST

8	Tissue homogenization buffer	0.1 M phosphate buffer	GPx
9	Tissue homogenization buffer	0.6% sulfosalicylic acid–Triton-X solution	GSH
10	Tissue homogenization buffer	tris–HCl buffer (0.02 M, pH 7.5)	ACP and ALP
11	Tissue homogenization buffer	0.1 M Tris EDTA (pH 8.0)	LDH
12	Tissue homogenization buffer	5.0 Mm potassium phosphate and 1.0 mM ethylenediamine tetra acetic acid (EDTA)	Δ5, 3β-HSD and 17β -HSD
13	Lysis buffer	10 mM Tris, 400 mM NaCl, 1 mM EDTA and 1% Triton X-100	DNA fragmentation assay
14	10X TE buffer	0.1M Tris-Hcl, 0.01M EDTA	DNA fragmentation assay
15	RIPA Buffer	150 mM NaCl, 50 mM Tris, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS	Western blot
16	Resolving buffer	3M Tris-HCL, pH 8.8	Western blot
17	Stacking buffer	0.5M Tris-HCL, pH 6.8	Western blot
18	Sample buffer	0.5M Tris-HCL, 12.5% Glycerol, 10% SDS, 0.05% (w/v) Bromophenol blue	Western blot
19	Running buffer		Western blot
20	Transfer buffer	0.25M Tris, 1.92M Glycine	Western blot
21	1X TBS-Tween-20	0.1% Tween-20, 150mM NaCl, 50mM Tris-HCL, pH 7.6	Western blot
22	10X AP substrate buffer	1M NaCl and 1M Tris-HCl, pH: 9.5	Western blot

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Document Information

Analyzed document	Priyankar_5000 words - Plagiarism checking.docx (D169246704)
Submitted	6/1/2023 9:40:00 AM
Submitted by	Rabishankar Giri
Submitter email	rabi.lib@presiuniv.ac.in
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Analysis address	rabi.lib.presi@analysis.urkund.com

Sources included in the report

Entire Document

Assessment of fluoride induced male reproductive disorders in adult Wistar rats and its amelioration by supplementation of vitamin C and E Submission of 5000 Words synopsis
Priyankar Pal Ph.D. registration No.: R-17RS18170107 Date: 14th August, 2018
Supervisor: Prof. Prabir Kumar Mukhopadhyay Reproductive and Environmental Toxicology Laboratory Department of Life Sciences Presidency University Kolkata- 700073, West Bengal, India
Synopsis (5000 words)
Name: Priyankar Pal
Supervisor: Prof. Prabir Kumar Mukhopadhyay
Title of Thesis: Assessment of fluoride induced male reproductive disorders in adult Wistar rats and its amelioration by supplementation of vitamin C and E
PhD registration Number with date: R-17RS18170107dated 14th August, 2018
Enrolment Number with date: PU/PHD/BIOS/2017/019 dated 20th September, 2017
Place of work: Presidency University, Kolkata
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Member Secretary
Dr. Prabir Kr. Mukhopadhyay
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IAEC-PU Ref: PU/IAEC/PM/20 Date: 29.4.2019

Dr. Prof. Prabir Kr. Mukhopadhyay
/ Dr.

Sub: Decision on Review of the Animal Experimentation Protocol

Title: Assessment of fluoride induced male reproductive disorders in adult Wistar rats and its amelioration by dietary supplements.

Study No. Study Site:

Dr.
/ Dr.

In its meeting held on the members of IAEC PU reviewed and discussed your animal study protocol for research project and other related documents for the proposed to your study entitled as above.

i) The following documents were reviewed:
a) Form B according to CPCSEA guidelines.
b) List of Principal Investigator and Co-investigators of the study.
c) Principal Investigators declaration.

ii) The following members of the committee were present

Prof. (Dr.) Devashish Sen	(Chairman)
Dr. Prabir kr. Mukhopadhyay	(Member Secretary)
Prof. (Dr.) Antony Gomes	(Main Nominee from CPCSEA)
Dr. Anjan Adhikari	(Biological Scientist from CPCSEA)
Mr. Dhruba Mukherjee	(Non-scientific socially aware member from CPCSEA)
Prof. Nirmal Kumar Sarkar	(Biological scientist Internal Member)
Dr. Amlan Ghosh	(Biological Scientist Internal Member)

TOXICOLOGY MECHANISMS AND METHODS
2021, VOL. 31, NO. 5, 383–392
https://doi.org/10.1080/15376516.2021.1891489

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RESEARCH ARTICLE

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Fluoride induced testicular toxicities in adult Wistar rats

Priyankar Pal and Prabir Kumar Mukhopadhyay

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ABSTRACT

Fluoride is essential for the development of teeth and bone but its excessive exposure causes reprotoxic effects. We have studied the graded effects of different doses of sodium fluoride (NaF) on 24 adult Wistar rats which were randomly divided into four groups ($n = 6$). All the rats were given normal diet prepared in our laboratory. Control (group I) rats were received vehicle only and treated rats (group II, III, and IV) were administered NaF orally at 10, 15, and 20 mg/kg/d doses, respectively, for 30 consecutive days. Gravimetric index of testis was decreased significantly in group III and IV rats but such changes were not observed in the accessory reproductive organs. Marked alterations in number, motility, viability, and plasma membrane functional integrity of caudal spermatozoa were noted in most of the treated groups. Noticeable alterations in testicular histoarchitecture were observed in group III and IV rats. Graded Changes of testicular redox homeostasis were noted by assessment of enzymatic (superoxide dismutase [SOD], catalase, glutathione s-transferase [GST], and glutathione peroxidase [GPx]) and nonenzymatic (malondialdehyde [MDA] and *protein carbonyl* [PC]) markers. Changes of testicular functional markers (acid phosphatase [ACP], alkaline phosphatase [ALP], and lactate dehydrogenase (LDH) activity) were noted in group III and IV rats. DNA fragmentation assay proved the possibility of DNA damage caused by oxidative stress, as evidenced by prominent trailing pattern of fragmented testicular genomic DNA from group III and IV rats. The study concludes that fluoride-mediated oxidative stress not only impedes the structural and functional facets of testis but also results in testicular DNA damage.

ARTICLE HISTORY
Received 3 January 2021
Revised 11 February 2021
Accepted 12 February 2021

KEYWORDS
Fluoride; testes; oxidative stress; functional alteration; DNA damage

1. Introduction

Fluorine is an electronegative trace element which is likewise considered as a pervasive environmental pollutant that subsists in rocks, water, soil, food, toothpaste, mouthwash, and other different sources of exposure (Doull et al. 2006). Unlike other toxicants, fluoride (simplest anion form of fluorine) has biphasic role because it acts as a preventive weapon in dental caries and it also has mitogenic effects on bone formation but increased exposure might result in serious toxic effects on animals and human beings (Spittle 2009). In accordance with World Health Organization (WHO), the desirable upper limit of fluoride in drinking water is 1.5 ppm (Parihar et al. 2013).

Fluorosis, a state of chronic toxicity owing to excessive fluoride intake is an endemic medical issue and it has been estimated that in India about 66.62 million people are at jeopardy due to such toxicity (Susheela 2006). Not only dental and skeletal fluorosis (Lu et al. 2014) are the outcome of excessive fluoride consumption but it also causes harmful effects on soft tissues and as well as different systems like respiratory, nervous, digestive, immune, and reproductive (Flores-Méndez et al. 2014; Han et al. 2015; Huo et al. 2016).

Literature survey concludes several research works with respect to fluoride insult on male reproductive machinery by using animal models and as well as on human beings. Epidemiological studies demonstrated that Indian populations, suffered from skeletal fluorosis, due to prolonged intake of fluoride contaminated water exceeding 1.5 mg/l and in some cases deficiency of serum testosterone was noted where the level of fluoride shoots up to 14.5 mg/l (Kumar and Susheela 1995). In Russia, similar observations were reported on workers exposed to cryolite (Na_3AlF_6), which is a white or colorless mineral (Tokar and Savchenko 1977). Another study on human populations in some parts of India, where ground water fluoride contents range from 1.0 to 6.5 mg/l exhibited decreased serum testosterone level (Michael et al. 1996). Male infertility among married peoples was also reported in high endemic areas of India having fluoride concentration 38.5 mg/l (Neelam et al. 1987). Besides alterations of hormonal level, the birth abnormalities along with miscarriage were also detected in populations belong to those contaminated areas (Michael et al. 1996).

Experiments on *in-vivo* animal model testified that excessive fluoride consumption was seen to be positively correlated with male reproductive disorders as evidence by impaired sperm parameters, anomalous testicular structure, and tainted serum testosterone level (Cao et al. 2016; Dong et al. 2016). Quite a few investigations affirmed that reactive oxygen species (ROS) mediated oxidative stress generation is the key player of chronic fluoride exposure which results in

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Unsolicited Review

HET

Human and Experimental Toxicology
2021, Vol. 40(12) 2025–2038
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DOI: 10.1177/09603271211021474
journals.sagepub.com/home/het
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Molecular perspective concerning fluoride and arsenic mediated disorders on epididymal maturation of spermatozoa: A concise review

Priyankar Pal, Sagnik Biswas and Prabir Kumar Mukhopadhyay

Abstract

Epididymis is a complex tubular structure of male reproductive system where spermatozoa undergo maturation and gain the fertilizing ability. Epididymal pseudostratified columnar epithelium with different cell types play imperative role by their secretory properties and enrich the luminal microenvironment necessary for achieving spermatozoal motility. During epididymal transit several secretory proteins like P26h, SPAG11, HSPD1 and many others are deposited on spermatozoal surface. At the same time spermatozoal proteins are also modified in this intraluminal milieu, which include cyritestin, fertilin, CE9 and others. Natural and anthropogenic activities disclose various environmental pollutants which affect different physiological systems of animals and human being. Likewise, reproductive system is also being affected. Fluoride causes structural alterations of caput and cauda segments of epididymis. Redox homeostasis and functional integrity are also altered due to diminished activities of SOD1, GR, Crisp2, Lrp2 and other important proteins. On the contrary arsenic affects mostly on cauda segment. Redox imbalance and functional amendment in epididymis have been observed with arsenic revelation as evidenced by altered genomic appearance of SOD, GST, catalase, Ddx3Y, VEGF and VEGFR2. This review is dealt with structure-function interplay in normal epididymal spermatozoal maturation along with subsequent complications developed under fluoride and arsenic toxicities.

Keywords

Epididymis, spermatozoal maturation, reprotoxicity, fluoride, arsenic

Introduction

Spermatogenesis is exclusively dependent on close liaison in-between Sertoli and Leydig cells of testis.[1] However, the spermatozoa formed by the testis are immature and infertile.[2,3] The spermatozoa need to be transported via epididymis to mature entirely and thus attain the competence to fertilize.[4,5] The epididymis is sustained by an array of homeostatic control systems operated by hormonal and non-hormonal features, which coalesce to ensure spermatozoal maturation and fortification. It is a unique and fascinating accessory sex organ of the male reproductive system made up of super coiled and convoluted tubule restraining a nutritious luminal fluid required by the spermatozoa for the attainment of fertility.[6,7] Structural orientation has sub-divided epididymis into three different regions like caput, corpus and cauda.[6] They are composed of various types of cells which in turn by their secretory activities help to create and maintain the luminal fluid composition for spermatozoal nourishment.[7] The cauda portion of the epididymis stores the matured spermatozoa and also plays an active role in spermatozoal nutrition and transportation. Thus, epididymal maturation of spermatozoa is

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BIOMARKERS
2022, VOL. 27, NO. 4, 361–374
https://doi.org/10.1080/1354750X.2022.2048891

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ORIGINAL ARTICLE

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Vitamin C and E supplementation can ameliorate NaF mediated testicular and spermatozoal DNA damages in adult Wistar rats

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ABSTRACT

Objective: Present study was designed to explore the efficacy of vitamin C and E (VC&VE) against fluoride mediated testicular, epididymal and spermatozoal anomalies.

Materials and methods: Thirty two adult Wistar rats were divided into four groups. Group-I was control; Group-II received sodium fluoride (NaF) at 15 mg/kg/day dose; Group-III was provided with VC (200 mg/kg/day) and VE (400 mg/kg/day) plus NaF; Group-IV received only VC&VE. Structural integrity and oxidative stress markers (superoxide dismutase, catalase, malondialdehyde and protein carbonyl) of testis and epididymis were assessed. Spermatozoal parameters (count, motility, viability and hypo-osmotic swelling) were evaluated. Testicular functional maker enzymes (acid phosphatase, alkaline phosphatase and lactate dehydrogenase) were also assessed. Integrity of testicular and spermatozoal DNA was evaluated. Testicular fluoride content was measured.

Result: Fluoride induced structural changes and alterations of oxidative stress markers were observed in testis and epididymis. Spermatozoal potentials were altered and reduced activities of testicular functional marker enzymes were observed. Fluoride caused testicular and spermatozoal DNA damages. VC&VE supplementation resulted in protection from all fluoride mediated alterations and helped in attenuating testicular fluoride accumulation.

Conclusion: Antioxidant properties of VC&VE ameliorated fluoride mediated reproductive damages but only supplementation did not exhibit any notable effect compared to control rats.

ARTICLE HISTORY
Received 25 July 2021
Accepted 27 February 2022

KEYWORDS
Fluoride; reproduction; DNA-damage; mitigation; vitamin-C; vitamin-E

Introduction

Fluoride, the ionic form of fluorine is a non-metallic, electronegative halogen and ubiquitously exists in biosphere. Apart from natural sources, several anthropogenic activities like water fluoridation, industrialisation, use of fluoride containing dental products and pesticides also cause environmental contamination with fluoride (Cengeloglu *et al.* 2002). The major sources through which humans are exposed to fluoride are fluoridated dental products and contaminated water (Zuo *et al.* 2018). After ingestion, fluoride is rapidly absorbed through stomach and small intestine via pH dependent and pH independent manners respectively, followed by distribution in skeletal and non skeletal tissues, and eventually the rest is excreted through urine (Palmer and Gilbert 2012). As per the World Health Organisation (WHO) guideline the permissible range of fluoride in drinking water is 0.5–1.5 ppm (WHO 2011).

Fluoride exerts self-contradictory effects because it plays an imperative role in teeth and bone formation by its mitogenic activities within safe limit, nevertheless, beyond upper limit it causes metabolic disruption leading to skeletal as well as nonskeletal fluorosis (Arif *et al.* 2012). Fluoride can cross the cell membrane and enters different soft tissues causing disorders in different systems including immune, nervous, hepatic, renal, digestive, respiratory and also reproductive (Guney *et al.* 2007).

Epidemiological study demonstrated that humans, when suffered from skeletal fluorosis for long time also experienced with significant reduction of serum testosterone level (Susheela and Jethanandani 1996). Male infertility was also reported among married peoples in some endemic areas of India where the ground water fluoride concentration was 38.5 mg/L (Neelam *et al.* 1987). Decreased serum testosterone level was also reported on Russian workers who were exposed to a fluorinated compound (cryolite) for more than 15 years (Ortiz-Pérez *et al.* 2003). Animal experimentations with overexposed fluoride had also shown altered testicular histomorphometry, impaired spermatozoal status and tainted steroidogenesis (Cao *et al.* 2016, Dong *et al.* 2016). Another recent report states that fluoride is also responsible for altered activities of testicular functional markers along with loss of testicular genomic DNA integrity (Pal and Mukhopadhyay 2021). Abnormalities and reduction of capacitation ability of epididymal spermatozoa were also disparaging outcome due to fluoride mediated decreased tyrosin phosphorylation and actin polymerisation of spermatozoa (Dvoráková-Hortová *et al.* 2008).

Excess fluoride is also responsible for the generation of reactive oxygen species (ROS) like superoxide anions,

Genes & Diseases xxx (xxxx) xxx

Available online at www.sciencedirect.com

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REVIEW ARTICLE

Molecular basis of fluoride toxicities: Beyond benefits and implications in human disorders

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Received 8 April 2022; received in revised form 10 August 2022; accepted 8 September 2022

KEYWORDS
Fluoride;
Autophagy;
Mitochondrial dysfunction;
Signaling cascades;
System disorders

Abstract Detrimental impacts of fluoride have become a global concern for several decades. Despite its beneficial role which is restricted only in skeletal tissues, deleterious effects are also observed in soft tissues and systems. The generation of enhanced oxidative stress is the commencement of excess fluoride exposure which may lead to cell death. Fluoride causes cell death through autophagy via Beclin 1 and mTOR signaling pathways. Beside these, several organ specific anomalies through different signaling pathways have been documented. Mitochondrial dysfunction, DNA damage, autophagy and apoptosis are the damaging outcomes in case of hepatic disorders. Urinary concentration defects and cell cycle arrest have been reported in renal tissues. Abnormal immune response has been characterized in the cardiac system. Cognitive dysfunction, neurodegenerative condition and learning impairment have also been

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